PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/86, 5/10, 7/04, 9/00, C07K 14/00, 14/005, 14/15, 14/54

A2

(11) International Publication Number: **WO 95/30763**

(43) International Publication Date: 16 November 1995 (16.11.95)

(21) International Application Number: PCT/US95/05789

(22) International Filing Date: 9 May 1995 (09.05.95)

(30) Priority Data:
08/240,030 9 May 1994 (09.05.94) US

(71) Applicant: VIAGENE, INC. [US/US]; 11055 Roselle Street, San Diego, CA 92121 (US).

(72) Inventor: RESPESS, James, G.; 4966 Lamont Street, San Diego, CA 92109 (US).

(74) Agents: McMASTERS, David, D. et al.; Seed and Berry, 6300 Columbia Center, 701 Fifth Avenue, Seattle, WA 98104-7092 (US).

(81) Designated States: AM, AU, BB, BG, BR, BY, CA, CN, CZ, EE, ES, FI, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LV, MD, MG, MN, MW, MX, NO, NZ, PL, RO, RU, SD, SG, SI, SK, TJ, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: RETROVIRAL VECTORS HAVING A REDUCED RECOMBINATION RATE

```
A TAT ATA TAT ATC GAT ACC  ATG GGG CAA ACC GTG ACT ACC CCT CTG TCC
                        ▶Met Gly Gln Thr Val Thr Thr Pro Leu Ser

CTC ACA CTG GGC CAT TGG AAG GAC GTG GAA AGA ATT GCC CAT AAT CAA AGC
▶Leu Thr Leu Gly His Trp Lys Asp Val Glu Arg Ile Ala His Asn Gln Ser

GTG GAC GTC AAA AAA CGC AGG TGG GTG ACA TTT TGT AGC GCC GAG TGG CCC
▶Val Asp Val Lys Lys Arg Arg Trp Val Thr Phe Cys Ser Ala Glu Trp Pro

ACA TTC AAT GTT GGC TGG CCT AGG GAT GGA ACT TTC AAT CGC GAT CTG ATT
▶Thr Phe Asn Val Gly Trp Pro Arg Asp Gly Thr Phe Asn Arg Asp Leu Ile

ACT CAA GTG AAA ATT AAA GTG TTC AGC CCC GGA CCC CAC GGC CAT CCC GAT
▶Thr Gln Val Lys Ile Lys Val Phe Ser Pro Gly Pro His Gly His Pro Asp

CAA GTT CCT TAT ATT GTC ACA TGG GAG GCT CTC GCT TTC GAT CCA CCA CCT
▶Gln Val Pro Tyr Ile Val Thr Trp Glu Ala Leu Ala Phe Asp Pro Pro Pro

TGG GTG AAA CCA TTC GTG CAT CCC AAA CCA CCT CCA CCC CTC CCA CCC AGC
▶Trp Val Lys Pro Phe Val His Pro Lys Pro Pro Pro Pro Leu Pro Pro Ser

GCT CCT AGC CTG CCC TTG GAG CCC CCA CGA AGC ACA CCA CCC AGG AGC AGC
▶Ala Pro Ser Leu Pro Leu Glu Pro Pro Arg Ser Thr Pro Pro Arg Ser Ser

                                    NarI
TTG TAC CCT GCT CTG ACC CCC AGC CTC GGC GCC AAA   CCT AAA C
▶Leu Tyr Pro Ala Leu Thr Pro Ser Leu Gly Ala Lys ? ????????
```

(57) Abstract

Retroviral vector constructs are described which have a 5' LTR, a tRNA binding site, a packaging signal, one or more heterologous sequences, an origin of second strand synthesis and a 3' LTR, wherein the vector construct lacks retroviral *gag/pol* or *env* coding sequences. In addition, *gag/pol*, and *env* expression cassettes are described wherein the expression cassettes lack a consecutive sequence of more than 8 nucleotides in common. The above-described retroviral vector constructs, *gag/pol* and *env* expression cassettes may be utilized to construct producer cell lines which preclude the formation of replication competent virus.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgyzstan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

Description

RETROVIRAL VECTORS HAVING A REDUCED RECOMBINATION RATE

Technical Field

The present invention relates generally to retroviral vectors for use in gene transfer, and more specifically, to retroviral vectors which are constructed such that the formation of replication competent virus by recombination is precluded.

Background of the Invention

Retroviruses are RNA viruses which can replicate and integrate into a host cell's genome through a DNA intermediate. This DNA intermediate, or provirus, may be stably integrated into the host's cellular DNA. Retroviruses are known to be responsible for a wide variety of diseases in both man and animals, including for example AIDS and a wide variety of cancers.

Although retroviruses can cause disease, they also have a number of properties that lead them to be considered as one of the most promising techniques for genetic therapy of disease. These properties include: (1) efficient entry of genetic material (the vector genome) into cells; (2) an active efficient process of entry into the target cell nucleus; (3) relatively high levels of gene expression; (4) minimal pathological effects on target cells; and (5) the potential to target particular cellular subtypes through control of the vector-target cell binding and tissue-specific control of gene expression. In using a retrovirus for genetic therapy, a foreign gene of interest may be incorporated into the retrovirus in place of normal retroviral RNA. When the retrovirus injects its RNA into a cell, the foreign gene is also introduced into the cell, and may then be integrated into the host's cellular DNA as if it were the retrovirus itself. Expression of this foreign gene within the host results in expression of foreign protein by the host cell.

Most retroviral vector systems which have been developed for gene therapy are based on murine retroviruses. Briefly, these retroviruses exist in two forms, as proviruses integrated into a host's cellular DNA, or as free virions. The virion form of the virus contains the structural and enzymatic proteins of the retrovirus (including reverse transcriptase), two RNA copies of the viral genome, and portions of the cell's plasma membrane in which is embedded the viral envelope glycoprotein. The genome is organized into four main regions: the Long Terminal Repeat (LTR), and the *gag*, *pol*, and *env* genes. The LTR may be found at both ends of the proviral genome, is a composite of the 5' and 3' ends of the RNA genome, and contains *cis*-acting elements

necessary for the initiation and termination of transcription. The three genes *gag, pol,* and *env* are located between the terminal LTRs. The *gag* and *pol* genes encode, respectively, internal viral structures and enzymatic proteins (such as integrase). The *env* gene encodes the envelope glycoprotein (designated gp70 and p15e) which confers infectivity and host range specificity of the virus, as well as the "R" peptide of undetermined function.

An important consideration in using retroviruses for gene therapy is the availability of "safe" retroviruses. Packaging cell lines and vector producing cell lines have been developed to meet this concern. Briefly, this methodology employs the use of two components, a retroviral vector and a packaging cell line (PCL). The retroviral vector contains long terminal repeats (LTRs), the foreign DNA to be transferred and a packaging sequence (ψ). This retroviral vector will not reproduce by itself because the genes which encode structural and envelope proteins are not included within the vector genome. The PCL contains genes encoding the *gag, pol,* and *env* proteins, but does not contain the packaging signal "ψ". Thus, a PCL can only form empty virion particles by itself. Within this general method, the retroviral vector is introduced into the PCL, thereby creating a vector-producing cell line (VCL). This VCL manufactures virion particles containing only the retroviral vector's (foreign) genome, and therefore has previously been considered to be a safe retrovirus vector for therapeutic use.

There are, however, several shortcomings with the current use of VCLs. One issue involves the generation of "live virus" (*i.e.*, replication competent retrovirus; RCR) by the VCL. Briefly, RCR can be produced in conventional producer cells when: (1) The vector genome and the helper genomes recombine with each other; (2) The vector genome or helper genome recombines with homologous cryptic endogenous retroviral elements in the producer cell; or (3) Cryptic endogenous retroviral elements reactivate (*e.g.*, xenotropic retroviruses in mouse cells).

Another issue is the propensity of mouse based VCLs to package endogenous retrovirus-like elements (which can contain oncogenic gene sequences) at efficiencies close to that with which they package the desired retroviral vector. Such elements, because of their retrovirus-like structure, are transmitted to the target cell to be treated at frequencies that parallel its transfer of the desired retroviral vector sequence.

A third issue is the ability to make sufficient retroviral vector particles at a suitable concentration to: (1) treat a large number of cells (*e.g.*, 10^8 - 10^{10}); and (2) manufacture vector particles at a commercially viable cost.

In order to construct safer PCLs, researchers have generated deletions of the 5' LTR and portions of the 3' LTR of helper elements (*see*, Miller and Buttimore, *Mol. Cell. Biol. 6*:2895-2902, 1986). When such cells are used, two recombination events are necessary to form the wild-type, replication competent genome. Nevertheless, results from several laboratories have indicated that even when several deletions are present, RCR may still be generated (*see*, Bosselman et al., *Mol. Cell. Biol. 7*:1797-1806, 1987; Danos and Mulligan, *Proc. Nat'l. Acad. Sci. USA 81*:6460-6464, 1988). In addition, cell lines containing both 5' and 3' LTR deletions which have been constructed have thus far not proven useful since they produce relatively low titers (Dougherty et al., *J. Virol. 63*:3209-3212, 1989).

One of the more recent approaches to constructing safer packaging cell lines involves the use of complementary portions of helper virus elements, divided among two separate plasmids, one containing *gag* and *pol*, and the other containing *env* (*see*, Markowitz et al., *J. Virol. 62*:1120-1124; and Markowitz et al., *Virology 167*:600-606, 1988. One benefit of this double-plasmid system is that three recombination events are required to generate a replication competent genome. Nevertheless, these double-plasmid vectors have also suffered from the drawback of including portions of the retroviral LTRs, and therefore remain capable of producing infectious virus.

The present invention overcomes the difficulties of recombination and low titer associated with many of the prior packaging cell lines, and further provides other related advantages.

Summary of the Invention

Briefly stated, the present invention provides compositions and methods for the construction of packaging cell lines which preclude the formation of RCR by homologous recombination. Within one aspect of the invention, recombinant retroviral vector constructs (RETROVECTOR™) are provided comprising a 5' LTR, a tRNA binding site, a packaging signal, one or more heterologous sequences, an origin of second strand DNA synthesis, and a 3' LTR, wherein the retroviral vector construct lacks *gag/pol* and *env* coding sequences. Within one embodiment of the invention, the retroviral vector construct lacks an extended packaging signal. Within one embodiment, the retroviral vector construct lacks a retroviral nucleic acid sequence upstream of the 5' LTR. Within a preferred embodiment, the retroviral vector constructs lack an *env* coding sequence upstream of the 5' LTR. Retroviral vector constructs of the present invention may be constructed from one or more retroviruses, including, for example, a

wide variety of amphotropic, ecotropic, xenotropic, and polytropic viruses (*see e.g.*, Figures 17A, B, and C).

As noted above, retroviral vector constructs of the present invention include one or more heterologous sequences. Within certain embodiments of the invention, the heterologous sequence is at least x kb in length, wherein x is selected from the group consisting of 1, 2, 3, 4, 5, 6, 7 and 8. Within one embodiment, the heterologous sequence is a gene encoding a cytotoxic protein, such as, for example, ricin, abrin, diphtheria toxin, cholera toxin, gelonin, pokeweed, antiviral protein, tritin, Shigella toxin, and Pseudomonas exotoxin A. Within other embodiments the heterologous sequence may be an antisense sequence, or an immune accessory molecule. Representative examples of immune accessory molecules include IL-1, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-13, and IL-14. Particularly preferred immune accessory molecules may be selected from the group consisting of IL-2, IL-12, IL-15 and gamma-interferon, or the group consisting of ICAM-1, ICAM-2, β-microglobin, LFA3, HLA class I and HLA class II molecules.

Within other embodiments of the invention, the heterologous sequence may encode a gene product that activates a compound with little or no cytotoxicity into a toxic product. Representative examples of such gene products include type I thymidine kinases such as HSVTK and VZVTK. Within another embodiment, the heterologous sequence may be a ribozyme. Within yet other embodiments, the heterologous sequence is a replacement gene, which encode proteins such as Factor VIII, ADA, HPRT, CF and the LDL Receptor. Within other embodiments, the heterologous sequence encodes an immunogenic portion of a virus selected from the group consisting of HBV, HCV, HPV, EBV, FeLV, FIV, and HIV.

Within other aspects of the present invention, *gag/pol* expression cassettes are provided, comprising a promoter operably linked to a *gag/pol* gene, and a polyadenylation sequence, wherein the *gag/pol* gene has been modified to contain codons which are degenerate for gag. Within one embodiment, the 5' terminal end of the *gag/pol* gene lacks a retroviral packaging signal sequence. Within other aspects *gag/pol* expression cassettes are provided comprising a promoter operably linked to a *gag/pol* gene, and a polyadenylation sequence, wherein the expression cassette does not co-encapsidate with a replication competent virus.

Within another aspect of the present invention, *gag/pol* expression cassettes are provided comprising a promoter operably linked to a *gag/pol* gene, and a polyadenylation sequence, wherein a 3' terminal end of the *gag/pol* gene has been deleted without effecting the biological activity of integrase. Within one embodiment, a

5' terminal end of the *gag/pol* gene has been modified to contain codons which are degenerate for gag. Within a further embodiment, the 5' terminal end of the *gag/pol* gene lacks a retroviral packaging signal sequence. Within other embodiments, the 3' terminal end has been deleted upstream (5') of nucleotide 5751 of SEQ ID NO: 1.

Within other aspects of the present invention, *env* expression cassettes are provided, comprising a promoter operably linked to an *env* gene, and a polyadenylation sequence, wherein no more than 6 retroviral nucleotides are included upstream of the *env* gene. Within another aspect, *env* expression cassettes are provided comprising a promoter operably linked to an *env* gene, and a polyadenylation sequence, wherein the *env* expression cassette does not contain a consecutive sequence of more than 8 nucleotides which are found in a *gag/pol* gene. Within yet another aspect, *env* expression cassettes are provided comprising a promoter operably linked to an *env* gene, and a polyadenylation sequence, wherein a 3' terminal end of the *env* gene has been deleted without effecting the biological activity of env. Within one embodiment, the 3' terminal end of the gene has been deleted such that a complete R peptide is not produced by the expression cassette. Within a further embodiment, the *env* gene is derived from a type C retrovirus, and the 3' terminal end has been deleted such that the *env* gene includes less than 18 nucleic acids which encode the R peptide. Within a preferred embodiment, the 3' terminal end has been deleted downstream from nucleotide 7748 of SEQ ID NO: 1.

Within various embodiments of the invention, the promoters of the *gag/pol* and *env* expression cassettes described above are heterologous promoters, such as CMV IE, the HVTK promoter, RSV promoter, Adenovirus major-later promoter and the SV40 promoter. Within other embodiments, the polyadenylation sequence is a heterologous polyadenylation sequence, such as the SV40 late poly A Signal and the SV40 early poly A Signal.

Within another aspect of the present invention, packaging cell lines are provided, comprising a *gag/pol* expression cassette and an *env* expression cassette, wherein the *gag/pol* expression cassette lacks a consecutive sequence of greater than 20, preferably greater than 15, more preferably greater than 10, and most preferably greater than 8 consecutive nucleotides which are found in the *env* expression cassette. Within other aspects, producer cell lines are provided comprising a *gag/pol* expression cassette, *env* expression cassette, and a retroviral vector construct, wherein the *gag/pol* expression cassette, *env* expression cassette and retroviral vector construct lack a consecutive sequence of greater than 20, preferably greater than 15, more preferably greater than 10, and most preferably greater than 8 nucleotides in common.

Representative examples of such retroviral vector constructs, *gag/pol* and *env* expression cassettes are described in more detail below.

Within yet another aspect of the present invention, producer cell lines are provided comprising a packaging cell line as described above, and a retroviral vector construct. Within another aspect of the present invention, producer cell lines are provided comprising a *gag/pol* expression cassette, *env* expression cassette and a retroviral vector construct, wherein the *gag/pol* expression cassette, *env* expression cassette and retroviral vector construct lack a consecutive sequence of greater than eight nucleotides in common.

Within other aspects of the invention, methods of producing a packaging cell line are provided, comprising the steps of (a) introducing a *gag/pol* expression cassette as described above into an animal cell; (b) selecting a cell containing a *gag/pol* expression cassette which expresses high levels of *gag/pol*, (c) introducing an *env* expression cassette into said selected cell, and (d) selecting a cell which expresses high levels of *env* and thereby producing the packaging cell. Within other aspects of the invention, the *env* expression cassette may be introduced into the cell first, followed by the *gag/pol* expression cassette. Within other aspects, methods are provided for producing recombinant retroviral particles comprising the step of introducing a retroviral vector construct into a packaging cell as described above. Within preferred embodiments, the retroviral vector construct is one of the retroviral vector constructs described above.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references are set forth below which describe in more detail certain procedures or compositions (*e.g.*, plasmids, etc.), and are therefore incorporated by reference in their entirety.

Brief Description of the Drawings

Figure 1 is a schematic illustration of pKS2+Eco57I-LTR(+).

Figure 2 is a schematic illustration of pKS2+Eco57I-LTR(-).

Figure 3 is a schematic illustration of pKS2+LTR-EcoRI.

Figure 4 is a schematic illustration of pR1.

Figure 5 is a schematic illustration of pR2.

Figure 6 is a schematic illustration of pKT1.

Figure 7 is a schematic illustration of pRI-HIVenv.

Figure 8 is a schematic illustration of pR2-HIVenv.

Figure 9 is a representative "prewobble" sequence for a MoMLV *gag/pol* (*see also* SEQ I.D. Nos. 11 and 12).

Figure 10 is a representative "wobble" sequence for a MoMLV *gag/pol* (*see also* SEQ. I.D. Nos. 9 and 10).

Figure 11 is a schematic illustration of pHCMV-PA.

Figure 12 is a schematic illustration of pCMV *gag/pol*.

Figure 13 is a schematic illustration of pCMVgpSma.

Figure 14 is a schematic illustration of pCMVgp-X.

Figure 15 is a schematic illustration of pCMV env-X.

Figure 16 is a schematic illustration of pRgpNeo.

Figures 17A, B and C comprise a table which sets forth a variety of retroviruses which may be utilized to construct the retroviral vector constructs, *gag/pol* expression cassettes and *env* expression cassettes of the present invention.

Figure 18 is a schematic illustration of pCMV Envam-Eag-X-less.

Figure 19A is a diagrammatic illustration of a "wobble" -*gag* construct. Figure 19B is a diagrammatic illustration of a "normal" -*gag* construct.

Detailed Description of the Invention

Prior to setting forth the invention, it may be helpful to an understanding thereof to first set forth definitions of certain terms that will be used hereinafter.

"Retroviral vector construct" refers to an assembly which is, within preferred embodiments of the invention, capable of directing the expression of a sequence(s) or gene(s) of interest. Briefly, the retroviral vector construct must include a 5' LTR, a tRNA binding site, a packaging signal, one or more heterologus sequences, an origin of second strand DNA synthesis and a 3' LTR. A wide variety of heterologous sequences may be included within the vector construct, including for example, sequences which encode a protein (*e.g.*, cytotoxic protein, disease-associated antigen, immune accessory molecule, or replacement gene), or which are useful as a molecule itself (*e.g.*, as a ribozyme or antisense sequence). Alternatively, the heterologous sequence may merely be a "stuffer" or "filler" sequence, which is of a size sufficient to allow production of viral particles containing the RNA genome. Preferably, the heterologous sequence is at least 1, 2, 3, 4, 5, 6, 7 or 8 kB in length.

The retroviral vector construct may also include transcriptional promoter/enhancer or locus defining element(s), or other elements which control gene expression by means such as alternate splicing, nuclear RNA export, post-translational modification of messenger, or post-transcriptional modification of protein. Optionally,

the retroviral vector construct may also include selectable markers such as Neo, TK, hygromycin, phleomycin, histidinol, or DHFR, as well as one or more specific restriction sites and a translation termination sequence.

"Expression cassette" refers to an assembly which is capable of directing the expression of the sequence(s) or gene(s) of interest. The expression cassette must include a promoter which, when transcribed, is operably linked to the sequence(s) or gene(s) of interest, as well as a polyadenylation sequence. Within preferred embodiments of the invention, both the promoter and the polyadenylation sequence are from a source which is heterologous to the helper elements (*i.e.*, *gag/pol* and *env*). Expression cassettes of the present invention may be utilized to express a *gag/pol* gene or an *env* gene. In addition, the expression cassettes may also be utilized to express one or more heterologous sequences either from a *gag/pol* and/or *env* expression cassette, or from a entirely different expression cassette.

Within preferred embodiments of the invention, the expression cassettes described herein may be contained within a plasmid construct. In addition to the components of the expression cassette, the plasmid construct may also include a bacterial origin of replication, one or more selectable markers, a signal which allows the plasmid construct to exist as single-stranded DNA (*e.g.*, a M13 origin of replication), a multiple cloning site, and a "mammalian" origin of replication (*e.g.*, a SV40 or adenovirus origin of replication).

PREPARATION OF RETROVIRAL VECTOR CONSTRUCTS, *GAG/POL* EXPRESSION CASSETTES AND *ENV* EXPRESSION CASSETTES

As noted above, the present invention provides compositions and methods for constructing packaging cells which preclude the formation of replication competent virus by homologous recombination. The following sections describe the preparation of retroviral vector constructs, *gag/pol* expression cassettes, and *env* expression cassettes.

1. *Construction of retroviral vector constructs*

Within one aspect of the present invention, retroviral vector constructs are provided comprising a 5' LTR, a tRNA binding site, a packaging signal, one or more heterologous sequences, an origin of second strand DNA synthesis and a 3' LTR, wherein the vector construct lacks *gag/pol* or *env* coding sequences. Briefly, Long Terminal Repeats ("LTRs") are subdivided into three elements, designated U5, R and U3. These elements contain a variety of signals which are responsible for the biological

activity of a retrovirus, including for example, promoter and enhancer elements which are located within U3. LTR's may be readily identified in the provirus due to their precise duplication at either end of the genome.

The tRNA binding site and origin of second strand DNA synthesis are also important for a retrovirus to be biologically active, and may be readily identified by one of skill in the art. For example, tRNA binds to a retroviral tRNA binding site by Watson-Crick base pairing, and is carried with the retrovirus genome into a viral particle. The tRNA is then utilized as a primer for DNA synthesis by reverse transcriptase. The tRNA binding site may be readily identified based upon its location just downstream from the 5' LTR. Similarly, the origin of second strand DNA synthesis is, as its name implies, important for the second strand DNA synthesis of a retrovirus. This region, which is also referred to as the poly-purine tract, is located just upstream of the 3' LTR.

In addition to 5' and 3' LTRs, a tRNA binding site, and an origin of second strand DNA synthesis, retroviral vector constructs of the present invention also comprise a packaging signal, as well as one or more heterologous sequences, each of which is discussed in more detail below.

Retroviral vector constructs of the present invention may be readily constructed from a wide variety of retroviruses, including for example, B, C, and D type retroviruses as well as spumaviruses and lentiviruses (*see* RNA Tumor Viruses, Second Edition, Cold Spring Harbor Laboratory, 1985). Briefly, viruses are often classified according to their morphology as seen under electron microscopy. Type "B" retroviruses appear to have an eccentric core, while type "C" retroviruses have a central core. Type "D" retroviruses have a morphology intermediate between type B and type C retroviruses. Representative examples of suitable retroviruses include those set forth below in Figures 17A, B and C (*see* RNA Tumor Viruses at pages 2-7), as well as a variety of xenotropic retroviruses (*e.g.*, NZB-X1, NZB-X2 and NZB_{9-1} (*see* O'Neill et al., *J. Vir. 53*:100-106, 1985)) and polytropic retroviruses (*e.g.*, MCF and MCF-MLV (*see* Kelly et al., *J. Vir. 45*(1):291-298, 1983)). Such retroviruses may be readily obtained from depositories or collections such as the American Type Culture Collection ("ATCC"; Rockville, Maryland), or isolated from known sources using commonly available techniques.

Particularly preferred retroviruses for the preparation or construction of retroviral vector constructs of the present invention include retroviruses selected from the group consisting of Avian Leukosis Virus, Bovine Leukemia Virus, Murine Leukemia Virus, Mink-Cell Focus-Inducing Virus, Murine Sarcoma Virus, Reticuloendotheliosis virus, Gibbon Ape Leukemia Virus, Mason Pfizer Monkey Virus,

and Rous Sarcoma Virus. Particularly preferred Murine Leukemia Viruses include 4070A and 1504A (Hartley and Rowe, *J. Virol. 19*:19-25, 1976), Abelson (ATCC No. VR-999), Friend (ATCC No. VR-245), Graffi, Gross (ATCC No. VR-590), Kirsten, Harvey Sarcoma Virus and Rauscher (ATCC No. VR-998), and Moloney Murine Leukemia Virus (ATCC No. VR-190). Particularly preferred Rous Sarcoma Viruses include Bratislava, Bryan high titer (*e.g.*, ATCC Nos. VR-334, VR-657, VR-726, VR-659, and VR-728), Bryan standard, Carr-Zilber, Engelbreth-Holm, Harris, Prague (*e.g.*, ATCC Nos. VR-772, and 45033), and Schmidt-Ruppin (*e.g.* ATCC Nos. VR-724, VR-725, VR-354).

Any of the above retroviruses may be readily utilized in order to assemble or construct retroviral vector constructs, packaging cells, or producer cells of the present invention given the disclosure provided herein, and standard recombinant techniques (*e.g.*, Sambrook et al, *Molecular Cloning: A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, 1989; Kunkle, *PNAS 82*:488, 1985). Further, within certain embodiments of the invention, portions of the retroviral vector construct may be derived from different retroviruses. For example, within one embodiment of the invention, retrovector LTRs may be derived from a Murine Sarcoma Virus, a tRNA binding site from a Rous Sarcoma Virus, a packaging signal from a Murine Leukemia Virus, and an origin of second strand synthesis from an Avian Leukosis Virus. Similarly, portions of a packaging cell line may be derived from different viruses (*e.g.*, a *gag/pol* expression cassette may be constructed from a Moloney Murine Leukemia Virus, and an *env* expression cassette from a Mason Pfizer Monkey virus).

As noted above, within various aspects of the present invention, retroviral vector constructs are provided which have packaging signals, and which lack both *gag/pol* and *env* coding sequences. As utilized within the context of the present invention, a packaging signal should be understood to refer to that sequence of nucleotides which is not required for synthesis, processing or translation of viral RNA or assembly of virions, but which is required in *cis* for encapsidation of genomic RNA (*see* Mann et al., *Cell 33*:153-159, 1983; RNA Tumor Viruses, Second Edition, *supra*). Further, as utilized herein, the phrase "lacks *gag/pol* or *env* coding sequences" should be understood to refer to retrovectors which contain less than 20, preferably less than 15, more preferably less than 10, and most preferably less than 8 consecutive nucleotides which are found in *gag/pol* or *env* genes, and in particular, within *gag/pol* or *env* expression cassettes that are used to construct packaging cell lines for the retroviral vector construct. Representative examples of such retroviral vector constructs are set forth in more detail below and in Example 1.

As an illustration, within one embodiment of the invention construction of retroviral vector constructs which lack *gag/pol* or *env* sequences may be accomplished by preparing retroviral vector constructs which lack an extended packaging signal. As utilized herein, the phrase "extended packaging signal" refers to a sequence of nucleotides beyond the minimum core sequence which is required for packaging, that allows increased viral titer due to enhanced packaging. As an example, for the Murine Leukemia Virus MoMLV, the minimum core packaging signal is encoded by the sequence (counting from the 5' LTR cap site) from approximately nucleotide 144 of SEQ. I.D. No. 1, up through the *Pst* I site (nucleotide 567 of SEQ. I.D. No. 1). The extended packaging signal of MoMLV includes the sequence beyond nucleotide 567 up through the start of the *gag/pol* gene (nucleotide 621), and beyond nucleotide 1040. Thus, within this embodiment retroviral vector constructs which lack extended packaging signal may be constructed from the MoMLV by deleting or truncating the packaging signal downstream of nucleotide 567.

Within other embodiments of the invention, retroviral vector constructs are provided wherein the packaging signal that extends into, or overlaps with, retroviral *gag/pol* sequence is deleted or truncated. For example, in the representative case of MoMLV, the packaging signal is deleted or truncated downstream of the start of the *gag/pol* gene (nucleotide 621 of SEQ ID NO: 1). Within preferred embodiments of the invention, the packaging signal is terminated at nucleotide 570, 575, 580, 585, 590, 595, 600, 610, 615 or 617 of SEQ ID NO: 1.

Within other aspects of the invention, retroviral vector constructs are provided which include a packaging signal that extends beyond the start of the *gag/pol* gene (*e.g.*, for MoMLV, beyond nucleotide 621 of SEQ ID NO: 1). When such retroviral vector constructs are utilized, it is preferable to utilize packaging cell lines for the production of recombinant viral particles wherein the 5' terminal end of the *gag/pol* gene in a *gag/pol* expression cassette has been modified to contain codons which are degenerate for *gag*. Such *gag/pol* expression cassettes are described in more detail below in section 2, and in Example 3.

Within other aspects of the present invention, retroviral vector constructs are provided comprising a 5' LTR, a tRNA binding site, a packaging signal, an origin of second strand DNA synthesis and a 3' LTR, wherein the retrovector plasmid construct does not contain a retroviral nucleic acid sequence upstream of the 5' LTR. As utilized within the context of the present invention, the phrase "does not contain a retroviral nucleic acid sequence upstream of the 5' LTR" should be understood to mean that the retrovector plasmid construct contains less than 20, preferably less than 15, more

preferably less than 10, and most preferably less than 8 consecutive nucleotides which are found in a retrovirus, and more specifically, in a retrovirus which is homologous to the retroviral vector construct, upstream of and/or contiguous with the 5' LTR. Within preferred embodiments, the retrovector plasmid constructs do not contain an *env* coding sequence (as discussed below) upstream of the 5' LTR. A particularly preferred embodiment of such retrovector plasmid constructs is set forth in more detail below in Example 1.

Within a further aspect of the present invention, retrovector plasmid constructs are provided comprising a 5' LTR, a tRNA binding site, a packaging signal, an origin of second strand DNA synthesis and a 3' LTR, wherein the retrovector plasmid construct does not contain a retroviral packaging signal sequence downstream of the 3' LTR. As utilized herein, the term "packaging signal sequence" should be understood to mean a sequence sufficient to allow packaging of the RNA genome. A representative example of such a retroviral vector construct is set forth in more detail below in Example 1.

2. *Construction of gag/pol expression cassettes*

As noted above, the present invention also provides a variety of *gag/pol* expression cassettes which, in combination with the retroviral vector constructs and *env* expression cassettes of the present invention, enable the construction of packaging cell lines and producer cell lines which preclude the formation of replication competent virus. Briefly, retroviral *gag/pol* genes contain a *gag* region which encodes a variety of structural proteins that make up the core matrix and nucleocapsid, and a *pol* region which contains genes which encode (1) a protease for the processing of *gag/pol* and *env* proteins, (2) a reverse transcriptase polymerase, (3) an RNase H, and (4) an integrase, which is necessary for integration of the retroviral provector into the host genome. Although retroviral *gag/pol* genes may be utilized to construct the *gag/pol* expression cassettes of the present invention, a variety of other non-retroviral (and non-viral) genes may also be utilized to construct the *gag/pol* expression cassette. For example, a gene which encodes retroviral RNase H may be replaced with genes which encode bacterial (*e.g.*, *E. coli* or *Thermus thermophilus*) RNase H. Similarly, a retroviral integrase gene may be replaced by other genes with similar function (*e.g.*, yeast retrotransposon TY3 integrase).

Within one aspect of the invention, *gag/pol* expression cassettes are provided comprising a promoter operably linked to a *gag/pol* gene, and a polyadenylation sequence, wherein the *gag/pol* gene has been modified to contain

codons which are degenerate for gag. Briefly, as noted above, in wild-type retrovirus the extended packaging signal of the retrovirus overlaps with sequences which encode gag and pol. Thus, in order to eliminate the potential of crossover between the retroviral vector construct and the *gag/pol* expression cassette, as well as to eliminate the possiblity of co-encapsidation of the *gag/pol* expression cassette and replication competent virus or retroviral vector constructs, sequences of overlap should be eliminated. Within one embodiment of the invention, elimination of such overlap is accomplished by modifying the *gag/pol* gene (and more specifically, regions which overlap with the retroviral vector construct, such as the extended packaging signal) to contain codons that are degenerate (*i.e.*, that "wobble") for gag. In particular, within preferred embodiments of the invention codons are selected which encode biologically active gag/pol protein (*i.e.*, capable of producing a competent retroviral particle, in combination with an *env* expressing element, and a RNA genome), and which lack any packaging signal sequence, including in particular, extended packaging signal sequence. As utilized herein, the phrase "lacks any retroviral packaging signal sequence" should be understood to mean that the *gag/pol* expression cassette contains less than 20, preferably less than 15, more preferably less than 10, and most preferably less than 8 consecutive nucleotides which are identical to a sequence found in a retroviral packaging signal (*e.g.*, in the case of MoMLV, extending up and through the *Xho* 1 site at approximately nucleotide number 1561). A particularly preferred example of such modified codons which are degenerate for gag is shown in Figure 10, and in Example 3, although the present invention should not be so limited. In particular, within other embodiments, at least 25, 50, 75, 100, 125 or 135 *gag* codons are modified or "wobbled" from the native *gag* sequence within the *gag/pol* expression cassettes of the present invention.

In addition to eliminating overlap between the retroviral vector construct and the *gag/pol* gene, it is also preferable to eliminate any potential overlap between the *gag/pol* gene and the *env* gene in order to prohibit the possibility of homologous recombination. This may be accomplished in at least two principal ways: (1) by deleting a portion of the *gag/pol* gene which encodes the integrase protein, and in particular, that portion of the gene which encodes the integrase protein which overlaps with the *env* coding sequence, or (2) by selecting codons which are degenerate for integrase and/or env.

Thus, within one aspect of the present invention *gag/pol* expression cassettes are provided comprising a promoter operably linked to a *gag/pol* gene, and a polyadenylation sequence or signal, wherein a 3' terminal end of the gene has been deleted without effecting the biological activity of the integrase. (The biological activity

of integrase may be readily determined by detection of an integration event, either by DNA analysis or by expression of a transduced gene; *see* Roth et al., *J. Vir.* *65*(4):2141-2145, 1991.) As an example, in the Murine Leukemia Virus MoMLV (SEQ ID. NO. 1), the *gag/pol* gene is encoded by nucleotides 621 through 5834. Within this sequence, the protein integrase is encoded by nucleotides 4610 through nucleotide 5834. A portion of the *gag/pol* sequence which encodes integrase also encodes env (which begins at nucleotide 5776). Thus, within one embodiment of the invention, the 3' terminal end of the *gag/pol* gene is deleted or truncated in order to prevent crossover with the *env* gene, without effecting the biological activity of the integrase. Within other preferred embodiments, the *gag/pol* gene is deleted at any nucleotide downstream (3') from the beginning of the integrase coding sequence, and preferably prior to the start of the *env* gene sequence. Within one embodiment, the sequence encoding gag/pol is a MoMLV sequence, and the *gag/pol* gene is deleted at any nucleotide between nucleotides 4610 and 5576 (of SEQ. I.D. No. 1), including for example, at nucleotides 5775, 5770, 5765, 5760, 5755, 5750.

Within other embodiments of the invention, the *gag/pol* expression cassette contains sequences encoding gag/pol (and including integrase), while lacking any sequence found in an *env* gene. The phrase "lacking any sequence found in an *env* gene" should be understood to mean that the *gag/pol* expression cassette does not contain at least 20, preferably at least 15, more preferably at least 10, and most preferably less than 8 consecutive nucleotides which are identical to an *env* sequence, and preferably which are found in an *env* expression cassette which will be utilized along with the *gag/pol* expression cassette to form a packaging cell. Such expression cassettes may be readily prepared by selecting codons which are degenerate for integrase, and which do not encode biologically active env. (*See Morgenstern and Land, Nuc. Acids Res. 18*:3587-3596, 1990.)

Within other embodiments of the invention, the *gag/pol* expression cassette contains a heterologous promoter, and/or heterologous polyadenylation sequence. As utilized herein, "heterologous" promoters or polyadenylation sequences refers to promoters or polyadenylation sequences which are from a different source from which the *gag/pol* gene (and preferably the *env* gene and retroviral vector construct) is derived from. Representative examples of suitable promoters include the Cytomegalovirus Immediate Early ("CMV IE") promoter, the Herpes Simplex Virus Thymidine Kinase ("HSVTK") promoter, the Rous Sarcoma Virus ("RSV") promoter, the Adenovirus major-late promoter and the SV 40 promoter. Representative examples

of suitable polyadenylation signals include the SV 40 late polyadenylation signal and the SV40 early polyadenylation signal.

Within preferred aspects of the present invention, *gag/pol* expression cassettes such as those described above will not co-encapsidate along with a replication competent virus. One representative method for determination of co-encapsidation is set forth below in Example 8.

3. *Construction of env expression cassettes*

Within other aspects of the present invention, *env* expression cassettes are provided which, in combination with the *gag/pol* expression cassettes and retroviral vector constructs described above, preclude formation of replication competent virus by homologous recombination, as well as to confer a particular specificity of the resultant vector particle (*e.g.*, amphotropic, ecotropic, xenotropic or polytropic; *see* Figure 17, as well as the discussion above). Briefly, in a wild-type retrovirus the *env* gene encodes two principal proteins, the surface glycoprotein "SU" and the transmembrane protein "TM", which are translated as a polyprotein, and subsequently separated by proteolytic cleavage. Representative examples of the SU and TM proteins are the gp120 protein and gp41 protein in HIV, and the gp70 protein and p15e protein in MoMLV. In some retroviruses, a third protein designated the "R" peptide" of undetermined function, is also expressed from the *env* gene and separated from the polyprotein by proteolytic cleavage. In the Murine Leukemia Virus MoMLV, the R peptide is designated "p2".

A wide variety of *env* expression cassettes may be constructed given the disclosure provided herein, and utilized within the present invention to preclude homologous recombination. Within one aspect of the present invention, *env* expression cassettes are provided comprising a promoter operably linked to an *env* gene, wherein no more than 6, 8, 10, 15, or 20 consecutive retroviral nucleotides are included upstream (5') of and/or contiguous with said *env* gene. Within other aspects of the invention, *env* expression cassettes are provided comprising a promoter operably linked to an *env* gene, wherein the *env* expression cassette does not contain a consecutive sequence of greater than 20, preferably less than 15, more preferably less than 10, and most preferably less than 8 or 6 consecutive nucleotides which are found in a *gag/pol* gene, and in particular, in a *gag/pol* expression cassette that will be utilized along with the *env* expression cassette to create a packaging cell line.

Within another aspect of the present invention, *env* expression cassettes are provided comprising a promoter operably linked to an *env* gene, and a polyadenylation sequence, wherein a 3' terminal end of the *env* gene has been deleted

without effecting the biological activity of env. As utilized herein, the phrase "biological activity of env" refers to the ability of envelop protein to be expressed on the surface of a virus or vector particle, and to allow for a successful infection of a host cell. One practical method for assessing biological activity is to transiently transfect the *env* expression cassette into a cell containing a previously determined functional *gag/pol* expression cassette, and a retroviral vector construct which expresses a selectable marker. A biologically functional *env* expression cassette will allow vector particles produced in that transfected cell, to transmit the selectable marker to a naive sensitive cell such that it becomes resistant to the marker drug selection. Within a preferred embodiment of the invention, the 3' terminal end of the *env* gene is deleted or truncated such that a complete R peptide is not produced by the expression cassette. In the representative example of MoMLV, sequence encoding the R peptide (which begins at nucleotide 7734) is deleted, truncated, or, for example, terminated by insertion of a stop codon at nucleotide 7740, 7745, 7747, 7750, 7755, 7760, 7765, 7770, 7775, 7780, or any nucleotide in between.

Within another aspect of the present invention, *env* expression cassettes are provided which contain a heterologous promoter, and/or heterologous polyadenylation sequence. As utilized herein, "heterologous" promoters or polyadenylation sequences refers to promoters or polyadenylation sequences which are from a different source from which the *gag/pol* gene (and preferably the *env* gene and retroviral vector construct) is derived from. Representative examples of suitable promoters include the CMV IE promoter, the HSVTK promoter, the RSV promoter, the Adenovirus major-late promoter and the SV 40 promoters. Representative examples of suitable polyadenylation signals include the SV 40 late polyadenylation signal and the SV40 early polyadenylation signal.

HETEROLOGOUS SEQUENCES

As noted above, the retroviral vector constructs, *gag/pol* expression cassettes, and *env* expression cassettes of the present invention may contain (and express) one or more heterologous sequences. A wide variety of heterologous sequences may be utilized within the context of the present invention, including for example, cytotoxic genes, antisense sequences, sequences which encode gene products that activate a compound with little or no cytotoxicity (*i.e.*, a "prodrug") into a toxic product, sequences which encode immunogenic portions of disease-associated antigens and sequences which encode immune accessory molecules. Representative examples of cytotoxic genes include the genes which encode proteins such as ricin (Lamb et al., *Eur.*

J. Biochem. 148:265-270, 1985), abrin (Wood et al., *Eur. J. Biochem. 198*:723-732, 1991; Evensen, et al., *J. of Biol. Chem. 266*:6848-6852, 1991: Collins et al., *J. of Biol. Chem. 265*:8665-8669, 1990; Chen et al., *Fed. of Eur. Biochem Soc. 309*:115-118, 1992), diphtheria toxin (Tweten et al., *J. Biol. Chem. 260*:10392-10394, 1985), cholera toxin (Mekalanos et al., *Nature 306*:551-557, 1983; Sanchez & Holmgren, *PNAS 86*:481-485, 1989), gelonin (Stirpe et al., *J. Biol. Chem. 255*:6947-6953, 1980), pokeweed (Irvin, *Pharmac. Ther. 21*:371-387, 1983), antiviral protein (Barbieri et al., *Biochem. J. 203*:55-59, 1982; Irvin et al., *Arch. Biochem. & Biophys. 200*:418-425, 1980; Irvin, *Arch. Biochem. & Biophys. 169*:522-528, 1975), tritin, Shigella toxin (Calderwood et al., *PNAS 84*:4364-4368, 1987; Jackson et al., *Microb. Path. 2*:147-153, 1987), and Pseudomonas exotoxin A (Carroll and Collier, *J. Biol. Chem. 262*:8707-8711, 1987).

Within further embodiments of the invention, antisense RNA may be utilized as a cytotoxic gene in order to induce a potent Class I restricted response. Briefly, in addition to binding RNA and thereby preventing translation of a specific mRNA, high levels of specific antisense sequences may be utilized to induce the increased expression of interferons (including gamma-interferon), due to the formation of large quantities of double-stranded RNA. The increased expression of gamma interferon, in turn, boosts the expression of MHC Class I antigens. Preferred antisense sequences for use in this regard include actin RNA, myosin RNA, and histone RNA. Antisense RNA which forms a mismatch with actin RNA is particularly preferred.

Within other embodiments of the invention, antisense sequences are provided which inhibit, for example, tumor cell growth, viral replication, or a genetic disease by preventing the cellular synthesis of critical proteins needed for cell growth. Examples of such antisense sequences include antisense thymidine kinase, antisense dihydrofolate reductase (Maher and Dolnick, *Arch. Biochem. & Biophys. 253*:214-220, 1987; Bzik et al., *PNAS 84*:8360-8364, 1987), antisense HER2 (Coussens et al., *Science 230*:1132-1139, 1985), antisense ABL (Fainstein, et al., *Oncogene 4*:1477-1481, 1989), antisense Myc (Stanton et al., *Nature 310*:423-425, 1984) and antisense *ras*, as well as antisense sequences which block any of the enzymes in the nucleotide biosynthetic pathway.

Within other aspects of the invention, retroviral vector constructs, *gag/pol* expression cassettes and *env* expression cassettes are provided which direct the expression of a gene product that activates a compound with little or no cytotoxicity (*i.e.*, a "prodrug") into a toxic product. Representative examples of such gene products include varicella zoster virus thymidine kinase (VZVTK), herpes simplex virus thymidine

kinase (HSVTK) (Field et al., *J. Gen. Virol. 49*:115-124, 1980; Munir et al., *Protein Engineering* 7(1):83-89, 1994; Black and Loeb, *Biochem 32*(43):11618-11626, 1993), and *E. coli.* guanine phosphoribosyl transferase (*see* U.S. Patent Application Serial No. 08/155,944, entitled "Compositions and Methods for Utilizing Conditionally Lethal Genes," filed November 18, 1993; *see also* WO 93/10218 entitled "Vectors Including Foreign Genes and Negative Selection Markers", WO 93/01281 entitled "Cytosine Deaminase Negative Selection System for Gene Transfer Techniques and Therapies", WO 93/08843 entitled "Trapped Cells and Use Thereof as a Drug", WO 93/08844 entitled "Transformant Cells for the Prophylaxis or Treatment of Diseases Caused by Viruses, Particularly Pathogenic Retroviruses", and WO 90/07936 entitled "Recombinant Therapies for Infection and Hyperproliferative Disorders.") Within preferred embodiments of the invention, the retroviral vector constructs direct the expression of a gene product that activates a compound with little or no cytotoxicity into a toxic product in the presence of a pathogenic agent, thereby affecting localized therapy to the pathogenic agent (*see* WO 94/13304).

Within one embodiment of the invention, retroviral vector constructs are provided which direct the expression of a HSVTK gene downstream, and under the transcriptional control of an HIV promoter (which is known to be transcriptionally silent except when activated by HIV tat protein). Briefly, expression of the tat gene product in human cells infected with HIV and carrying the vector construct causes increased production of HSVTK. The cells (either *in vitro* or *in vivo*) are then exposed to a drug such as ganciclovir, acyclovir or its analogues (FIAU, FIAC, DHPG). Such drugs are known to be phosphorylated by HSVTK (but not by cellular thymidine kinase) to their corresponding active nucleotide triphosphate forms. Acyclovir and FIAU triphosphates inhibit cellular polymerases in general, leading to the specific destruction of cells expressing HSVTK in transgenic mice (*see* Borrelli et al., *Proc. Natl. Acad. Sci. USA 85*:7572, 1988). Those cells containing the recombinant vector and expressing HIV tat protein are selectively killed by the presence of a specific dose of these drugs.

Within further aspects of the present invention, retroviral vector constructs, *gag/pol* expression cassettes and *env* expression cassettes of the present invention may also direct the expression of one or more sequences which encode immunogenic portions of disease-associated antigens. As utilized within the context of the present invention, antigens are deemed to be "disease-associated" if they are either associated with rendering a cell (or organism) diseased, or are associated with the disease-state in general but are not required or essential for rendering the cell diseased. In addition, antigens are considered to be "immunogenic" if they are capable, under

appropriate conditions, of causing an immune response (either cell-mediated or humoral). Immunogenic "portions" may be of variable size, but are preferably at least 9 amino acids long, and may include the entire antigen.

A wide variety of "disease-associated" antigens are contemplated within the scope of the present invention, including for example immunogenic, non-tumorigenic forms of altered cellular components which are normally associated with tumor cells (*see* WO 93/10814). Representative examples of altered cellular components which are normally associated with tumor cells include ras* (wherein "*" is understood to refer to antigens which have been altered to be non-tumorigenic), p53*, Rb*, altered protein encoded by Wilms' tumor gene, ubiquitin*, mucin, protein encoded by the DCC. APC, and MCC genes, as well as receptors or receptor-like structures such as neu, thyroid hormone receptor, Platelet Derived Growth Factor ("PDGF") receptor, insulin receptor, Epidermal Growth Factor ("EGF") receptor, and the Colony Stimulating Factor ("CSF") receptor.

"Disease-associated" antigens should also be understood to include all or portions of various eukaryotic, prokaryotic or viral pathogens. Representative examples of viral pathogens include the Hepatitis B Virus ("HBV") and Hepatitis C Virus ("HCV"; *see* WO 93/15207), Human Papiloma Virus ("HPV"; *see* WO 92/05248; WO 90/10459; EPO 133,123), Epstein-Barr Virus ("EBV"; *see* EPO 173,254; JP 1,128,788; and U.S. Patent Nos. 4,939,088 and 5,173,414), Feline Leukemia Virus ("FeLV"; *see* WO 93/09070; EPO 377,842; WO 90/08832; WO 93/09238), Feline Immunodeficiency Virus ("FIV"; U.S. Patent No. 5,037,753; WO 92/15684; WO 90/13573; and JP 4,126,085), HTLV I and II, and Human Immunodeficiency Virus ("HIV"; *see* WO 93/02805).

Within other aspects of the present invention, the retroviral vector constructs, *gag/pol* expression cassettes and *env* expression cassettes described above may also direct the expression of one or more immune accessory molecules. As utilized herein, the phrase "immune accessory molecules" refers to molecules which can either increase or decrease the recognition, presentation or activation of an immune response (either cell-mediated or humoral). Representative examples of immune accessory molecules include α interferon, β interferon, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7 (U.S. Patent No. 4,965,195), IL-8, IL-9, IL-10, IL-11, IL-12 (Wolf et al., *J. Immun. 46*:3074, 1991; Gubler et al., *PNAS 88*:4143, 1991; WO 90/05147; EPO 433,827), IL-13 (WO 94/04680), IL-14, IL-15, GM-CSF, M-CSF-1, G-CSF, CD3 (Krissanen et al., *Immunogenetics 26*:258-266, 1987), CD8, ICAM-1 (Simmons et al., *Nature 331*:624-627, 1988), ICAM-2 (Singer, *Science 255*: 1671, 1992), β-microglobulin (Parnes et al.,

PNAS 78:2253-2257, 1981), LFA-1 (Altmann et al., *Nature 338*: 521, 1989), LFA3 (Wallner et al., *J. Exp. Med. 166*(4):923-932, 1987), HLA Class I, HLA Class II molecules, B7 (Freeman et al., *J. Immun. 143*:2714, 1989), and B7-2. Within a preferred embodiment, the heterologous gene encodes gamma-interferon.

Within preferred aspects of the present invention, the retroviral vector constructs described herein may direct the expression of more than one heterologous sequence. Such multiple sequences may be controlled either by a single promoter, or preferably, by additional secondary promoters (*e.g.*, Internal Ribosome Binding Sites or "IRBS"). Within preferred embodiments of the invention, retroviral vector constructs direct the expression of heterologous sequences which act synergistically. For example, within one embodiment retroviral vector constructs are provided which direct the expression of a molecule such as IL-15, IL-12, IL-2, gamma interferon, or other molecule which acts to increase cell-mediated presentation in the T_H1 pathway, along with an immunogenic portion of a disease-associated antigen. In such embodiments, immune presentation and processing of the disease-associated antigen will be increased due to the presence of the immune accessory molecule.

Within other aspects of the invention, retroviral vector constructs are provided which direct the expression of one or more heterologous sequences which encode "replacement" genes. As utilized herein, it should be understood that the term "replacement genes" refers to a nucleic acid molecule which encodes a therapeutic protein that is capable of preventing, inhibiting, stabilizing or reversing an inherited or noninherited genetic defect. Representative examples of such genetic defects include disorders in metabolism, immune regulation, hormonal regulation, and enzymatic or membrane associated structural function. Representative examples of diseases caused by such defects include Cystic Fibrosis ("CF"; *see* Dorin et al., *Nature 326*:614,), Parkinson's Disease, Adenosine Deaminase deficiency ("ADA"; Hahma et al., *J. Bact. 173*:3663-3672, 1991), β-globin disorders, Hemophilia A & B (Factor VIII-deficiencies; *see* Wood et al., *Nature 312*:330, 1984), Gaucher disease, diabetes, forms of gouty arthritis and Lesch-Nylan disease (due to "HPRT" deficiencies; *see* Jolly et al., *PNAS 80*:477-481, 1983) and Familial Hypercholesterolemia (LDL Receptor mutations; *see* Yamamoto et al., *Cell 39*:27-38, 1984).

Sequences which encode the above-described heterologous genes may be readily obtained from a variety of sources. For example, plasmids which contain sequences that encode immune accessory molecules may be obtained from a depository such as the American Type Culture Collection (ATCC, Rockville, Maryland), or from commercial sources such as British Bio-Technology Limited (Cowley, Oxford England).

Representative sources sequences which encode the above-noted immune accessory molecules include BBG 12 (containing the GM-CSF gene coding for the mature protein of 127 amino acids), BBG 6 (which contains sequences encoding gamma interferon), ATCC No. 39656 (which contains sequences encoding TNF), ATCC No. 20663 (which contains sequences encoding alpha interferon), ATCC Nos. 31902, 31902 and 39517 (which contains sequences encoding beta interferon), ATCC No 67024 (which contains a sequence which encodes Interleukin-1), ATCC Nos. 39405, 39452, 39516, 39626 and 39673 (which contains sequences encoding Interleukin-2), ATCC Nos. 59399, 59398, and 67326 (which contain sequences encoding Interleukin-3), ATCC No. 57592 (which contains sequences encoding Interleukin-4), ATCC Nos. 59394 and 59395 (which contain sequences encoding Interleukin-5), and ATCC No. 67153 (which contains sequences encoding Interleukin-6). It will be evident to one of skill in the art that one may utilize either the entire sequence of the protein, or an appropriate portion thereof which encodes the biologically active portion of the protein.

Alternatively, known cDNA sequences which encode cytotoxic genes or other heterologous genes may be obtained from cells which express or contain such sequences. Briefly, within one embodiment mRNA from a cell which expresses the gene of interest is reverse transcribed with reverse transcriptase using oligo dT or random primers. The single stranded cDNA may then be amplified by PCR (*see* U.S. Patent Nos. 4,683,202, 4,683,195 and 4,800,159. *See also* PCR Technology: Principles and Applications for DNA Amplification, Erlich (ed.), Stockton Press, 1989 all of which are incorporated by reference herein in their entirety) utilizing oligonucleotide primers complementary to sequences on either side of desired sequences. In particular, a double stranded DNA is denatured by heating in the presence of heat stable Taq polymerase, sequence specific DNA primers, ATP, CTP, GTP and TTP. Double-stranded DNA is produced when synthesis is complete. This cycle may be repeated many times, resulting in a factorial amplification of the desired DNA.

Sequences which encode the above-described genes may also be synthesized, for example, on an Applied Biosystems Inc. DNA synthesizer (*e.g.*, ABI DNA synthesizer model 392 (Foster City, California)).

PREPARATION OF RETROVIRAL PACKAGING CELL LINES, AND GENERATION OF RECOMBINANT VIRAL PARTICLES

As noted above, the *gag/pol* expression cassettes and *env* expression cassettes of the present invention may be used to generate transduction competent retroviral vector particles by introducing them into an appropriate parent cell line in

order to create a packaging cell line, followed by introduction of a retroviral vector construct, in order to create a producer cell line (*see* WO 92/05266). Such packaging cell lines, upon introduction of an N2-type vector construct (Armentano et al., *J. of Vir. 61*(5):1647-1650, 1987) produce a titer of greater than 10^5 cfu/ml, and preferably greater than 10-fold, 20-fold, 50-fold, or 100-fold higher titer than similar transduced PA317 cells (Miller and Buttimore, *Mol. and Cell. Biol. 6*(8):2895-2902, 1986).

Within one aspect of the present invention, methods for creating packaging cell lines are provided, comprising the steps of (a) introducing a *gag/pol* expression cassette according into an animal cell, (b) selecting a cell containing a *gag/pol* expression cassette which expresses high levels of *gag/pol*, (c) introducing an *env* expression cassette into the selected cell, and (d) selecting a cell which expresses high levels of *env*, and thereby creating the packaging cell. Within other aspects of the present invention, methods for creating packaging cell lines are provided comprising the steps of (a) introducing an *env* expression cassette into an animal cell (b) selecting a cell which expresses high levels of *env*, (c) introducing a *gag/pol* expression cassette into the selected cell, and (d) selecting a cell containing a *gag/pol* expression cassette which expresses high levels of *gag/pol*, and thereby creating the packaging cell. As utilized herein, it should be understood that "high" levels of gag/pol or env refers to packaging cells which produce at least z times greater gag/pol or env protein than PA317 cells, wherein z is selected from the group consisting of 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10.

A wide variety of animal cells may be utilized to prepare the packaging cells of the present invention, including for example human, macaque, dog, rat and mouse cells. Particularly preferred cell lines for use in the preparation of packaging cell lines of the present invention are those that lack genomic sequences which are homologous to the retroviral vector construct, *gag/pol* expression cassette and *env* expression cassette to be utilized. Methods for determining homology may be readily accomplished by, for example, hybridization analysis (*see Martin et al., PNAS 78*:4892-4896, 1981; *see also* WO 92/05266).

Expression cassettes of the present invention may be introduced into cells by numerous techniques, including for example, transfection by various physical methods, such as electroporation, DEAE dextran, lipofection (Felgner et al., *Proc. Natl. Acad. Sci. USA 84*:7413-7417, 1989), direct DNA injection (Acsadi et al., *Nature 352*:815-818, 1991); microprojectile bombardment (Williams et al., *PNAS 88*:2726-2730, 1991), liposomes of several types (*see e.g.*, Wang et al., *PNAS 84*:7851-7855, 1987); $CaPO_4$ (Dubensky et al., *PNAS 81*:7529-7533, 1984), DNA ligand (Wu et al, *J. of Biol. Chem. 264*:16985-16987, 1989), administration of nucleic acids alone (WO

90/11092), or administration of DNA linked to killed adenovirus (Curiel et al., *Hum. Gene Ther.* 3: 147-154, 1992).

Producer cell lines (also called vector-producing lines or "VCLs") may then be readily prepared by introducing a retroviral vector construct as described above, into a packaging cell line. Within preferred embodiments of the invention, producer cell lines are provided comprising a *gag/pol* expression cassette, an *env* expression cassette, and a retroviral vector construct, wherein the *gag/pol* expression cassette, *env* expression cassette and retroviral vector construct lack a consecutive sequence of greater than 20, preferably 15, more preferably 10, and most preferably 10 or 8 nucleotides in common.

PHARMACEUTICAL COMPOSITIONS

Within another aspect of the invention, pharmaceutical compositions are provided, comprising a recombinant viral particle as described above, in combination with a pharmaceutically acceptable carrier or diluent. Such pharmaceutical compositions may be prepared either as a liquid solution, or as a solid form (*e.g.*, lyophilized) which is suspended in a solution prior to administration. In addition, the composition may be prepared with suitable carriers or diluents for topical administration, injection, or oral, nasal, vaginal, sub-lingual, inhalant or rectal administration.

Pharmaceutically acceptable carriers or diluents are nontoxic to recipients at the dosages and concentrations employed. Representative examples of carriers or diluents for injectable solutions include water, isotonic saline solutions which are preferably buffered at a physiological pH (such as phosphate-buffered saline or Tris-buffered saline), mannitol, dextrose, glycerol, and ethanol, as well as polypeptides or proteins such as human serum albumin. A particularly preferred composition comprises a retroviral vector construct or recombinant viral particle in 10 mg/ml mannitol, 1 mg/ml HSA, 20 mM Tris, pH 7.2, and 150 mM NaCl. In this case, since the recombinant vector represents approximately 1 mg of material, it may be less than 1% of high molecular weight material, and less than 1/100,000 of the total material (including water). This composition is stable at -70°C for at least six months.

Pharmaceutical compositions of the present invention may also additionally include factors which stimulate cell division, and hence, uptake and incorporation of a recombinant retroviral vector. Representative examples include Melanocyte Stimulating Hormone (MSH), for melanomas or epidermal growth factor for breast or other epithelial carcinomas.

Particularly preferred methods and compositions for preserving recombinant viruses are described in U.S. applications entitled "Methods for Preserving Recombinant Viruses" (*see* WO 94/11414).

METHODS OF ADMINISTRATION

Within other aspects of the present invention, methods are provided for inhibiting or destroying pathogenic agents in a warm-blooded animal, comprising administering to a warm-blooded animal a recombinant viral particle as described above, such that the pathogenic agent is inhibited or destroyed. Within various embodiments of the invention, recombinant viral particles may be administered *in vivo*, or *ex vivo*. Representative routes for *in vivo* administration include intradermally ("i.d."), intracranially ("i.c."), intraperitoneally ("i.p."), intrathecally ("i.t."), intravenously ("i.v."), subcutaneously ("s.c."), intramuscularly ("i.m.") or even directly into a tumor.

Alternatively, the cytotoxic genes, antisense sequences, gene products, retroviral vector constructs or viral particles of the present invention may also be administered to a warm-blooded animal by a variety of other methods. Representative examples include transfection by various physical methods, such as lipofection (Felgner et al., *Proc. Natl. Acad. Sci. USA 84*:7413-7417, 1989), direct DNA injection (Acsadi et al., *Nature 352*:815-818, 1991); microprojectile bombardment (Williams et al., *PNAS 88*:2726-2730, 1991); liposomes of several types (*see e.g.*, Wang et al., *PNAS 84*:7851-7855, 1987); $CaPO_4$ (Dubensky et al., *PNAS 81*:7529-7533, 1984); DNA ligand (Wu et al, *J. of Biol. Chem. 264*:16985-16987, 1989); administration of nucleic acids alone (WO 90/11092); or administration of DNA linked to killed adenovirus (Curiel et al., *Hum. Gene Ther. 3*: 147-154, 1992).

Within a preferred aspect of the present invention, retroviral particles (or retroviral vector constructs alone) may be utilized in order to directly treat pathogenic agents such as a tumor. Within preferred embodiments, the retroviral particles or retroviral vector constructs described above may be directly administered to a tumor, for example, by direct injection into several different locations within the body of tumor. Alternatively, arteries which serve a tumor may be identified, and the vector injected into such an artery, in order to deliver the vector directly into the tumor. Within another embodiment, a tumor which has a necrotic center may be aspirated, and the vector injected directly into the now empty center of the tumor. Within yet another embodiment, the retroviral vector construct may be directly administered to the surface of the tumor, for example, by application of a topical pharmaceutical composition

containing the retroviral vector construct, or preferably, a recombinant retroviral particle.

Within another aspect of the present invention, methods are provided for inhibiting the growth of a selected tumor in a warm-blooded animal, comprising the steps of (a) removing tumor cells associated with the selected tumor from a warm-blooded animal, (b) infecting the removed cells with a retroviral vector construct which directs the expression of at least one anti-tumor agent, and (c) delivering the infected cells to a warm-blooded animal, such that the growth of the selected tumor is inhibited by immune responses generated against the gene-modified tumor cell. Within the context of the present invention, "inhibiting the growth of a selected tumor" refers to either (1) the direct inhibition of tumor cell division, or (2) immune cell mediated tumor cell lysis, or both, which leads to a suppression in the net expansion of tumor cells. Inhibition of tumor growth by either of these two mechanisms may be readily determined by one of ordinary skill in the art based upon a number of well known methods (*see* U.S. Serial No. 08/032,846). Examples of compounds or molecules which act as anti-tumor agents include immune accessory molecules, cytotoxic genes, and antisense sequences as discussed above (*see also* U.S. Serial No. 08/032,846).

Cells may be removed from a variety of locations including, for example, from a selected tumor. In addition, within other embodiments of the invention, a vector construct may be inserted into non-tumorigenic cells, including for example, cells from the skin (dermal fibroblasts), or from the blood (*e.g.*, peripheral blood leukocytes). If desired, particular fractions of cells such as a T cell subset or stem cells may also be specifically removed from the blood (*see*, for example, PCT WO 91/16116, an application entitled "Immunoselection Device and Method"). Vector constructs may then be contacted with the removed cells utilizing any of the above-described techniques, followed by the return of the cells to the warm-blooded animal, preferably to or within the vicinity of a tumor. Within one embodiment of the present invention, subsequent to removing tumor cells from a warm-blooded animal, a single cell suspension may be generated by, for example, physical disruption or proteolytic digestion. In addition, division of the cells may be increased by addition of various factors such as melanocyte stimulating factor for melanomas or epidermal growth factor for breast carcinomas, in order to enhance uptake, genomic integration and expression of the recombinant viral vector.

Within the context of the present invention, it should be understood that the removed cells may not only be returned to the same animal, but may also be utilized to inhibit the growth of selected tumor cells in another, allogeneic, animal. In such a

case it is generally preferable to have histocompatibility matched animals (although not always, *see, e.g.*, Yamamoto et al., "Efficacy of Experimental FIV Vaccines," 1st International Conference of FIV Researchers, University of California at Davis, September 1991).

The above-described methods may additionally comprise the steps of depleting fibroblasts or other non-contaminating tumor cells subsequent to removing tumor cells from a warm-blooded animal, and/or the step of inactivating the cells, for example, by irradiation.

As noted above, within certain aspects of the present invention, several anti-tumor agents may be administered either concurrently or sequentially, in order to inhibit the growth of a selected tumor in accordance with the methods of the present invention. For example, within one embodiment of the invention, an anti-tumor agent such as γ-IFN may be co-administered or sequentially administered to a warm-blooded animal along with other anti-tumor agents such as IL-2, or IL-12, in order to inhibit or destroy a pathogenic agent. Such therapeutic compositions may be administered directly utilizing a single vector construct which directs the expression of at least two anti-tumor agents, or, within other embodiments, expressed from independent vector constructs. Alternatively, one anti-tumor agent (*e.g.*, γ-IFN) may be administered utilizing a vector construct, while other tumor agents (*e.g.*, IL-2) are administered directly (*e.g.*, as a pharmaceutical composition intravenously).

Within a particularly preferred embodiment, retroviral vector constructs which deliver and express both a γ-IFN gene and another gene encoding IL-2, may be administered to the patient. In such constructs, one gene may be expressed from the retrovector LTR and the other may utilize an additional transcriptional promoter found between the LTRs, or may be expressed as a polycistronic mRNA, possibly utilizing an internal ribosome binding site. After *in vivo* gene transfer, the patient's immune system is activated due to the expression of γ-IFN. Infiltration of the dying tumor with inflammatory cells, in turn, increases immune presentation and further improves the patient's immune response against the tumor.

Within other aspects of the present invention, methods are provided for generating an immune response against an immunogenic portion of an antigen, in order to prevent or treat a disease (*see, e.g.*, U.S. Serial Nos. 08/104,424; 08/102,132, 07/948,358; 07/965,084), for suppressing graft rejection, (*see* U.S. Serial No. 08/116,827), for suppressing an immune response (*see* U.S. Serial No. 08/116,828), and for suppressing an autoimmune response (*see* U.S. Serial No. 08/116,983).

As will be understood by one of ordinary skill in the art given the disclosure provided herein, any of the retroviral vector constructs described herein may be delivered not only as a recombinant viral particle, but as direct nucleic acid vectors. Such vectors may be delivered utilizing any appropriate physical method of gene transfer, including for example, those which have been discussed above.

The following examples are offered by way of illustration, and not by way of limitation.

EXAMPLE 1

CONSTRUCTION OF RETROVECTOR BACKBONES

A. Preparation of a Retroviral vector construct That Does Not Contain an Extended Packaging Sequence (Ψ)

This example describes the construction of a retroviral vector construct using site-specific mutagenesis. Within this example, a MoMLV retroviral vector construct is prepared wherein the packaging signal "Ψ" of the retrovector is terminated at basepair 617 of SEQ ID NO: 1, thereby eliminating the ATG start of *gag*. Thus, no crossover can occur between the retroviral vector construct and the *gag/pol* expression cassette which is described below in Example 3.

Briefly, pMLV-K (Miller, *J. Virol* 49:214-222, 1984 - an infectious clone derived from pMLV-1 Shinnick et al., *Nature, 293*:543-548, 1981) is digested with *Eco*57I, and a 1.9kb fragment is isolated. (*Eco*57I cuts upstream from the 3' LTR, thereby removing all *env* coding segments from the retroviral vector construct.) The fragment is then blunt ended with T4 polymerase (New England Biolabs), and all four deoxynucleotides, and cloned into the *Eco*RV site of phagemid pBluescript II KS+ (Stratagene, San Diego, Calif.). This procedure yields two constructs, designated pKS2+Eco57I-LTR(+) (Figure 1) and pKS2+Eco57I-LTR(-) (Figure 2), which are screened by restriction analysis. When the (+) single stranded phagemid is generated, the sense sequence of MoMLV is isolated.

A new *Eco*RI site is then created in construct pKS2+*Eco*57I-LTR(+) in order to remove the ATG start codon of *gag*. In particular, an *Eco*RI site is created using the single stranded mutagenesis method of Kunkle (*PNAS 82*:488, 1985). pKS2+*Eco*57I-LTR(+) is a pBluescript™ II + phagemid (Strategene, San Diego, Calif.) containing an *Eco*57I fragment from pMLV-K. It includes the MoMLV LTR and downstream sequence to basepair 1378. When single stranded phagemid is generated the sense sequence of MoMLV is isolated. The oligonucleotide, 5'-GGT AAC AGT CTG GCC CGA ATT CTC AGA CAA ATA CAG (SEQ ID NO: 2), is created and used to generate an *Eco*RI site at basepairs 617-622. This construct is designated pKS2+LTR-EcoRI (Figure 3).

B. Substitution of Nonsense Codons in the Extended Packaging Sequence (Ψ+)

This example describes modification of the extended packaging signal (Ψ+) by site-specific mutagenesis. In particular, the modification will substitute a stop

codon, TAA, at the normal ATG start site of *gag* (position 631-633 of SEQ ID NO: 1), and an additional stop codon TAG at position 637-639 of SEQ ID NO: 1.

Briefly, an *Eco*57I - *Eco*RI fragment (MoMLV basepairs 7770 to approx. 1040) from pN2 (Amentano et al., J. Virol. 61:1647-1650, 1987) is first cloned into pBluescript II KS+ phagemid at the *Sac*II and *Eco*RI sites (compatible). Single stranded phagemid containing antisense MoMLV sequence, is generated using helper phage M13K07 (Stratagene, San Diego, Calif.). The oligonucleotide 5'-CTG TAT TTG TCT GAG AAT TAA GGC TAG ACT GTT ACC AC (SEQ ID NO: 3) is synthesized, and utilize according to the method of Kunkle as described above, in order to modify the sequence within the Ψ region to encode stop codons at nucleotides 631-633 and 637-639.

C. Removal of Retroviral Packaging Sequence Downstream from the 3' LTR

Retroviral packaging sequence which is downstream from the 3' LTR is deleted essentially as described below. Briefly, pKS2+Eco57I-LTR(-) (Figure 2) is digested with *Ball* and *Hinc*II, and relegated excluding the *Ball* to *Hinc*II DNA which contains the packaging region of MoMLV.

D. Construction of Vector Backbones

Constructs prepared in sections A and C above, or alternatively from sections B and C above, are combined with a plasmid vector as described below, in order to create a retrovector backbone containing all elements required *in cis*, and excluding all sequences of 8 nucleic acids or more contained in the retroviral portion of the *gag-pol* and *env* expression elements (*see* Examples 3 and 4).

1. Parts A and C are combined as follows: The product of A is digested with *Nhe*I and *Eco*RI, and a 1034 basepair fragment containing the LTR and minimal Ψ is isolated. The fragment is ligated into the product of part C at the unique (compatible) restriction sites *Spe*I and *Eco*RI. The resultant construct is designated pR1 (Figure 4)

2. Parts B and C are combined as follows: The product of B is digested with *Nhe*I and *Eco*RI and a 1456 basepair fragment containing the LTR and modified Ψ+ region is isolated. The fragment is ligated into the product of C at the unique (compatible) restriction sites *Spe*I and *Eco*RI. The resultant construct is designated pR2 (Figure 5).

EXAMPLE 2

INSERTION OF A GENE OF INTEREST INTO pR1 AND pR2

This example describes the insertion of a gene of interest, gp120, gp41, and rev along with a selectable marker into either pR1 or pR2. Briefly, the sequence encoding gp120, gp41 and rev is taken from construct pKT1 (Figure 6; *see also* Chada et al., *J. Vir. 67*:3409-3417, 1993); note that this vector is also referred to as N2IIIBenv. In particular, pKT1 is first digested at the unique *Asn*II site (position 5959). The ends are blunted, and an *Xho* I linker is ligated at that site. (New England Biolabs). The construct is then digested with *Xho* I, and a 4314 bp fragment containing HIV envelope (gp120 and gp41), rev, SV40 early promoter and G418 resistance genes is isolated.

pR1 or pR2 is digested at the unique *Eco* R1 restriction site, blunted, and *Sal* I linkers (New England Biolabs) are ligated in. The 4314 bp KT1 fragment is then ligated into pR1 or pR2 at the new *Sal* I sites, and the correct orientation is determined (*see* Figures 7 and 8). In both of these constructs, (pR1-HIVenv and pR2-HIVenv) the HIV genes are expressed from the MLV LTR, and G418 resistance is expressed from the SV40 promoter.

EXAMPLE 3

CONSTRUCTION OF *GAG-POL* EXPRESSION CASSETTES

A. Construction of an Expression Cassette Backbone, pHCMU-PA

A vector is first created in order to form the backbone for both the *gag/pol* and *env* expression cassettes. Briefly, pBluescript SK- phagemid (Stratagene, San Diego, Calif.; GenBank accession number 52324; referred to as "SK-") is digested with *Spe*I and blunt ended with Klenow. A blunt end *Dra*I fragment of SV40 (Fiers et al., "Complete nucleotide sequence of SV40 DNA" *Nature 273*:113-120, 1978) from DraI (bp 2366) to DraI (bp2729) is then inserted into SK-, and a construct isolated in which the SV40 late polyadenylation signal is oriented opposite to the LacZ gene of SK-. This construct is designated SK-SV40A.

A Human Cytomegalovirus Major Immediate Early Promoter ("HCMV-IE"; Boshart et al., *Cell 41*:521-530, 1985) (*Hin*cII, bp 140, to *Eag*I, bp814) is isolated after digestion with *Hin*cII and *Eag*I, and the *Eag*I site blunt ended. The 674 blunt ended fragment is ligated into SK-SV40A. The final construct, designated pHCMV-PA is then isolated (*see* Figure 11). This construct contains the HCMV promoter oriented

in opposite orientation to the LacZ gene, and upstream from the late polyadenylation signal of SV40.

B. Creation of New Codons for the 5' *Gag*

This example describes *gag/pol* expression cassettes that lack non-coding sequences upstream from the *gag* start, thereby reducing recombination potential between the *gag-pol* expression element and Ψ+ sequence of a retroviral vector construct, and inhibiting co-packaging of the *gag-pol* expression element along with the retrovector. In order to construct such an expression cassette, 448 bp of DNA is synthesized with the following features: 5' ATATATATATCGAT(*Cla*I site)ACCATG(start codon, position 621) (SEQ ID NO: 4), followed by 410 bp encoding 136+ amino acid residues using alternative codons (*see* Figures 9 and 10), followed by GGCGCC(*Nar*1 site)AAACCTAAAC 3' (SEQ ID NO: 5).

Briefly, each of oligos 15 through 24 (set forth below in Table 1) are added to a PCR reaction tube such that the final concentration for each is 1 μM. Oligos 25 and 26 are added to the tube such that the final concentration for each is 3 μM. 1.2 μL of 2.5 mM stock deoxynucleotide triphosphates (dG, dA, dT, dC) are added to the tube. 5 μL of 10X PCR buffer (Perkin Elmer). Water is added to a final volume of 50 μL. Wax beads are added and melted over the aqueous layer at 55°C and then cooled to 22°C. A top aqueous layer is added as follows: 5 μL 10X PCR buffer, 7.5 μL dimethylsulfoxide, 1.5 μL Taq polymerase (Perkin-Elmer) and 36 μL water. Forty cycles of PCR are then performed as follows: 94°C, 30 seconds; 56°C, 30 seconds; and 72°C, 30 seconds. The PCR product is stored at -20°C until assembly of the *gag/pol* expression cassette.

Table 1

SEQ. ID. No.	Sequence
15	5' ATA TAT ATA TAT CGA TAC CAT GGG GCA AAC CGT GAC TAC CCC TCT GTC CCT CA C ACT GGC CCA A 3'
16	5' TTG ATT ATG GGC AAT TCT TTC CAC GTC CTT CCA ATG GCC CAG TGT GAG GGA C 3'
17	5' AGA ATT GCC CAT AAT CAA AGC GTG GAC GTC AAA AAA CGC AGG TGG GT G ACA TTT TGT AGC GCC GAG TGG CCC 3'

18 5' AAG TTC CAT CCC TAG GCC AGC CAA CAT TGA ATG TGG GCC ACT CGG CGC
TAC A 3'

19 5' GGC CTA GGG ATG GAA CTT TCA ATC GCG ATC TGA TTA CTC AAG TGA AA A TTA AAG TGT TCA GCC CCG GAC CCC 3'

20 5' GTG ACA ATA TAA GGA ACT TGA TCG GGA TGG CCG TGG GGT CCG GGG CTG
AAC A 3'

21 5' AGT TCC TTA TAT TGT CAC ATC GGA GGC TCT CGC TTT CGA TCC ACC ACC TTG GGT GAA ACC ATT CGT GCA TCC 3'

22 5' AGG AGC GCT GGG TGG GAG GGG TGG AGG TGG TTT GGG ATG CAC GAA TGG TTT C 3'

23 5' CTC CCA CCC AGC GCT CCT AGC CTG CCC TTG GAG CCC CCA CGA AGC ACA CCA CCC AGG AGC AGC TTG TAC CCT 3'

24 5' GTT TAG GTT TGG CGC CGA GGC TGG GGG TCA GAG CAG GGT ACA AGC TGC
TCC T 3'

25 5' ATA TAT ATA TAT CGA TAC C 3'

26 5' GTT TAG GTT TGG CGC CGA GG 3'

C. Creation of a New 3' End for *Pol*

In order to prepare a *gag/pol* expression cassette which expresses full length *gag/pol*, pCMV*gag/pol* is constructed. Briefly, MoMLV sequence from *Pst*1 (BP567) to *Nhe*1 (bp 7847) is cloned into the *Pst*1-*Xba*1 sites of pUC19 (New England Biolabs). The resultant intermediate is digested with *Hin*dIII and *Xho*I, and a 1008 bp fragment containing the *gag* leader sequence is isolated. The same intermediate is also digested with *Xho*I and *Sca*I, and a 4312 bp fragment containing the remaining *gag* and *pol* sequences is isolated. The two isolated fragments are then cloned into the *Hin*dIII and *Sma*I sites of pHCMV-PA, described above. The resultant construct, designated CMV *gag/pol* (Figure 12) expresses MoMLV *gag* and *pol* genes.

In order to truncate the 3' end of the *pol* gene found in pCMV *gag-pol*, a 5531 basepair *Sna*B1 - *Xma*I fragment containing a portion of the CMV IE promoter and all of *gag-pol* except the final 28 codons, is isolated from pCMV *gag-pol*. This fragment is cloned into the *Sna*B1 and *Xma*I sites of pHCMV-PA. This construct expresses five new amino acids at the carboxy-terminus (Ser-Lys-Asn-Tyr-Pro) (SEQ ID NO: 6) (pCMV gpSma).

Alternatively, these five amino acids may be eliminated by digesting pCMVgp *Sma*I with *Sma*I and adding an *Nhe*I (termination codons in three phases) linker (5' - CTA GCT AGC TAG SEQ ID NO: 14; New England Biolabs) at the end of the truncated *pol* sequence. This construct is designated pCMV gp Nhe. Both of these constructs eliminates potential crossover between *gag/pol* and *env* expression cassettes.

D. *Gag-Pol* Expression Cassette

Parts B and C from above are combined to provide an expression vector containing a CMV IE promoter, *gag-pol* sequence starting from the new *Cla*I site (followed by ACC ATG and 412 bp of alternative or "wobble" *gag* coding sequence) and terminating at the *Sma*I site (MoMLV position 5750) followed by an SV40 polyadenylation signal, essentially as described below. Briefly, the approximately 451 bp double stranded wobble fragment from part A is ligated into pCR™II TA cloning vector (Invitrogen Corp.). The wobble PCR product naturally contains a 3' A-overhang at each end, allowing for cloning into the 3' T-overhang of pCR™II. The 422 bp *Cla*I -*Nar*I wobble fragment from the pCR™II clone is removed and is ligated into the *Cla*I (Position 679, Figure pCMV gp Sma) and *Nar*I (Position 1585) sites of pCMVgp Sma I (Part B) (or pCMV gp Nhe). (The *Cla*I site at position 5114 is methylated and not cut with *Cla*I). The product of that ligation is digested with *Nar*I, and the MLV-K *Nar*I fragment (positions 1035 to 1378) is inserted (SEQ ID NO: 1). This construct is designated pCMVgp -X (Figure 14).

EXAMPLE 4

CONSTRUCTION OF *ENV* EXPRESSION CASSETTES

A. Creation of a New 5' *Eag*I Restriction Site

Starting with an *Eag*I- *Eco*RI 626 bp subfragment from a 4070A amphotropic envelope (Chattopodhyay et al., *J. Vir. 39*:777, 1981; GenBank accession # MLV4070A, and #MLVENVC; SEQ ID NO: 12) cloned in a pBluescript II Ks+ vector (containing the start codon), site directed mutagenesis is performed upstream of the translation start site in order to change ACCATCCTCTGGACGGACATG... (SEQ ID NO: 7; positions 20 - 40 of Genebank sequence # MLVENVC) to ACCCGGCCGTGGACGGACATG... (SEQ ID NO: 8) and create a new *Eag*I site at position 23. This modification allows cloning of the amphotropic envelope sequence into an expression vector eliminating upstream 4070A sequence homologous to the *gag-pol* expression element as described in Example 2A.

B. Creation of a New 3' End for Env

A new 3' end of the envelope expression element is created by terminating the sequence which encodes the R-peptide downstream from the end of the transmembrane region (p15E). Briefly, construct pHCMV-PA, described above, is first modified by digestion with *Not*I (position 1097), blunted and relegated to obliterate the overlapping Bluescript *Eag*I site at the same position. pCMV Envam-Eag-X-less is then constructed by digesting the modified pHCMV-PA with *Eag*I (position 671 and *Sma*I (position 712) and ligating in two fragments. The first is an *EagI-NcoI* fragment from 4070A (positions 1-1455) (SEQ ID NO: 12). The second is an MLV-K envelope fragment, *NcoI - Pvu*II (positions 7227-7747) (SEQ ID NO: 12). The resultant construct from the three-way ligation contains the HCMV promoter followed by the SU (GP70) coding sequence of the 4070A envelope, the TM (p15e) coding sequence of MoMLV, and sequence encoding 8 residues of the R-peptide. In addition, this envelope expression cassette (pCMV Env am-Eag-X-less) (Figure 18) shares no sequence with crossless retrovector backbones described in Example 1.

C. Envelope Expression Element

Parts A and B from above are combined to complete an amphotropic expression element containing the CMV promoter, 4070A SU, MoMLV TM and SV40 polyadenylation signal in a Bluescript SK- plasmid vector. This construct is called pCMVenv-X (Figure 15). Briefly, the construct described in part A with a new *Eag*I restriction site is digested with *Eag*I and *Xho*I, and a 571 bp fragment is isolated. pCMV Envam-Eag-X-less (from part B) is digested with *Kpn*I and *Eag*I and the 695 bp fragment is reserved. pCMV Envam-Eag-X-less (from part B) is digested with *Kpn*I and *Xho*I and the 4649 bp fragment is reserved. These two fragments are ligated together along with the 571 bp *Eag*I to *Xho*I fragment digested from the PCR construct from part A. pCMVenv-X shares no sequence with crossless retrovector backbones nor the *gag-pol* expression element pCMVgp-X.

EXAMPLE 5

FUNCTIONALITY TESTS FOR *GAG-POL* AND *ENV* EXPRESSION CASSETTES

Rapid tests have been developed in order to ensure that the *gag-pol* and *env* expression cassettes are biologically active. The materials for these tests consist of a cell line used for transient expression (typically 293 cells, ATCC #CRL 1573), a target

cell line sensitive to infection (typically HT 1080 cells, ATCC #CCL 121) and either pRgpNeo (Figure 16) or pLARNL (Emi et al., *J. Virol 65*:1202-1207, 1991). The two later plasmids express rescuable retrovectors that confer G418 resistance and also express *gag-pol*, in the case of RgpNeo or *env*, in the case of pLARNL. For convenience, the organization of RgpNeo (Figure 16) is set forth below.

In order to test expression cassettes such as pCMVgp-X for functionality of *gag/pol*, the plasmid is co-transfected with pLARNL at a 1:1 ratio into 293 cells. After 12 hours, the media is replaced with normal growth media. After an additional 24 hours, supernatant fluid is removed from the 293 cells, filtered through a 0.45 μm filter, and placed on HT 1080 target cells. Twenty-four hours after that treatment, the media is replaced with growth media containing 800 ug/ml G418. G418 resistant colonies are scored after one week. The positive appearance of colonies indicates that all elements are functional and active in the original co-transfection.

For convenience, the organization of RgpNeo (Figure 16) is set forth below:
Position 1 = left end of 5' LTR; Positions 1-6320 = MoMLV sequence from 5'LTR to Sca 1 restriction site; Positions 6321 - 6675 = SV40 early promoter; Positions 6676-8001 = Neo resistance gene from Tn 5 (including prokaryotic promoter); and Positions 8002 - 8606 = pBR origin of replication.

EXAMPLE 6

PACKAGING CELL LINE AND PRODUCER CELL LINE DEVELOPMENT

This example describes the production of packing and producer cell lines utilizing the above described retroviral vector constructs, *gag/pol* expression cassettes, and *env* expression cassettes, which preclude the formation of replication competent virus.

Briefly, for amphotropic MoMLV-based retroviral vector constructs, a parent cell line is selected which lacks sequences which are homologous to Murine Leukemia Viruses, such as the dog cell line D-17 (ATCC No. CCL 183). The *gag/pol* expression cassettes are then introduced into the cell by electroporation, along with a selectable marker plasmid such as DHFR (Simonsen et al., *PNAS 80*:2495-2499, 1983). Resistant colonies are then selected, expanded in 6 well plates to confluency, and assayed for expression of gag/pol by Western Blots. Clones are also screened for the production of high titer vector particles after transduction with pLARNL.

The highest titer clones are then electroporated with an *env* expression cassette and a selectable marker plasmid such as hygromycin (*see* Gritz and Davies, *Gene 25*:179-188, 1983). Resistant colonies are selected, expanded in 6 well plates to confluency, and assayed for expression of env by Western Blots. Clones are also screened for the production of high titer vector particles after transduction with a retroviral vector construct.

Resultant packaging cell lines may be stored in liquid Nitrogen at 10 x 10^6 cells per vial, in DMEM containing 10% irradiated Fetal Bovine Serum, and 8% DMSO. Further testing may be accomplished in order to confirm sterility, and lack of helper virus production. Preferably, both an S+L- assay and a *Mus dunni* marker rescue assay should be performed in order to confirm a lack of helper virus production.

In order to construct a producer cell line, retroviral vector construct as described above in Example 1 is electroporated into a xenotropic packaging cell line made utilizing the methods described above. After 24-48 hours, supernatant fluid is removed from the xenotropic packaging cell line, and utilized to transduce a second packaging cell line, thereby creating the final producer cell line.

EXAMPLE 7

HELPER DETECTION ASSAY COCULTIVATION, AND MARKER RESCUE

This example describes a sensitive assay for the detection of replication competent retrovirus ("RCR"). Briefly, 5 x 10^5 vector-producing cells are cocultivated with an equal number of *Mus dunni* cells (Lander and Chattopadhyay, *J. Virol. 52*:695, 1984). *Mus dunni* cells are particularly preferred for helper virus detection because they are sensitive to nearly all murine leukemia-related viruses, and contain no known endogenous viruses. At three, six, and nine days after the initial culture, the cells are split approximately 1 to 10, and 5 x 10^5 fresh *Mus dunni* cells are added. Fifteen days after the initial cocultivation of *Mus dunni* cells with the vector-producing cells, supernatant fluid is removed from cultures, filtered through a 0.45 μm filter, and subjected to a marker rescue assay.

Briefly, culture fluid is removed from a MdH tester cell line (*Mus dunni* cells containing pLHL (a hygromycin resistance marker retroviral vector; *see* Palmer et al., *PNAS 84*(4):1055-1059, 1987) and replaced with the culture fluid to be tested. Polybrene is added to a final concentration of 4 μg/ml. On day 2, medium is removed and replaced with 2 ml of fresh DMEM containing 10% Fetal Calf Serum. On day 3, supernatant fluid is removed, filtered, and transferred to HT1080 cells. Polybrene is

added to a final concentration of 4μg/ml. On day 4, medium in the HT1080 cells is replaced with fresh DMEM containing 10% Fetal Calf Serum, and 100 μg/ml hygromycin. Selection is continued on days 5 through 20 until hygromycin resistant colonies can be scored, and all negative controls (*e.g.*, mock infected MdH cells) are dead.

EXAMPLE 8

ASSAY FOR ENCAPSIDATION OF WOBBLE RNA SEQUENCE

This example describes a sensitive assay for the detection of encapsidation of RNA from constructs containing wobble or normal gag sequence. Briefly, a fragment of DNA from a "wobble" *gag/pol* expression cassette (Example 3), containing the CMV promoter and gag sequence to the *Xho*I site (MoMLV position 1561) is ligated to a SV40 neo-3' LTR DNA fragment from N2 (Armentano et al., *supra*) or KT-3 (*see* WO 91/02805 or WO 92/05266). This construct is diagrammatically illustrated in Figure 19A, and is not expected to be encapsidated in packaging cell lines such as DA or HX (*see* WO 92/05266) because it lacks a 5' LTR and primer binding site.

A second construct is also made, similar to the first except that the wobble sequence is replaced by normal *gag* sequence. Similar to the first construct, the RNA transcribed from this DNA is not expected to be encapsidated. This construct is diagrammatically illustrated in Figure 19B.

The above constructs are separately transfected into a packaging cell line. The culture is then assayed for the ability to generate transducible G418-resistant retrovector. Neither construct results in transducible vector.

Cell cultures containing the above constructs are then transduced with the retrovector LHL (*see* Example 7). The cell cultures, after selection, will now generate retrovector conferring hygromycin resistance to target cells. Further, if co-encapsidation is allowed by interaction between LHL RNA and the transcripts from the above constructs, statistically significant RT-mediated recombination can occur resulting in the transfer of G418 resistance to target cells.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration,

various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Respess, James

(ii) TITLE OF INVENTION: Crossless Retroviral Vectors

(iii) NUMBER OF SEQUENCES: 26

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Seed & Berry
(B) STREET: 6300 Columbia Center; 701 Fifth Avenue
(C) CITY: Seattle
(D) STATE: Washington
(E) COUNTRY: USA
(F) ZIP: 98104

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: McMasters, David D.
(B) REGISTRATION NUMBER: 33,963
(C) REFERENCE/DOCKET NUMBER: 930049.424C1

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (206)622-4900
(B) TELEFAX: (206)682-6031

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8332 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GCGCCAGTCC TCCGATTGAC TGAGTCGCCC GGGTACCCGT GTATCCAATA AACCCTCTTG 60

CAGTTGCATC CGACTTGTGG TCTCGCTGTT CCTTGGGAGG GTCTCCTCTG AGTGATTGAC 120

TACCCGTCAG CGGGGGTCTT TCATTTGGGG GCTCGTCCGG GATCGGGAGA CCCCTGCCCA 180
GGGACCACCG ACCCACCACC GGGAGGTAAG CTGGCCAGCA ACTTATCTGT GTCTGTCCGA 240
TTGTCTAGTG TCTATGACTG ATTTTATGCG CCTGCGTCGG TACTAGTTAG CTAACTAGCT 300
CTGTATCTGG CGGACCCGTG GTGGAACTGA CGAGTTCGGA ACACCCGGCC GCAACCCTGG 360
GAGACGTCCC AGGGACTTCG GGGGCCGTTT TTGTGGCCCG ACCTGAGTCC AAAAATCCCG 420
ATCGTTTTGG ACTCTTTGGT GCACCCCCCT TAGAGGAGGG ATATGTGGTT CTGGTAGGAG 480
ACGAGAACCT AAAACAGTTC CCGCCTCCGT CTGAATTTTT GCTTTCGGTT TGGGACCGAA 540
GCCGCGCCGC GCGTCTTGTC TGCTGCAGCA TCGTTCTGTG TTGTCTCTGT CTGACTGTGT 600
TTCTGTATTT GTCTGAGAAT ATGGGCCAGA CTGTTACCAC TCCCTTAAGT TTGACCTTAG 660
GTCACTGGAA AGATGTCGAG CGGATCGCTC ACAACCAGTC GGTAGATGTC AAGAAGAGAC 720
GTTGGGTTAC CTTCTGCTCT GCAGAATGGC CAACCTTTAA CGTCGGATGG CCGCGAGACG 780
GCACCTTTAA CCGAGACCTC ATCACCCAGG TTAAGATCAA GGTCTTTTCA CCTGGCCCGC 840
ATGGACACCC AGACCAGGTC CCCTACATCG TGACCTGGGA AGCCTTGGCT TTTGACCCCC 900
CTCCCTGGGT CAAGCCCTTT GTACACCCTA AGCCTCCGCC TCCTCTTCCT CCATCCGCCC 960
CGTCTCTCCC CCTTGAACCT CCTCGTTCGA CCCCGCCTCG ATCCTCCCTT TATCCAGCCC 1020
TCACTCCTTC TCTAGGCGCC AAACCTAAAC CTCAAGTTCT TTCTGACAGT GGGGGGCCGC 1080
TCATCGACCT ACTTACAGAA GACCCCCGC CTTATAGGGA CCCAAGACCA CCCCCTTCCG 1140
ACAGGGACGG AAATGGTGGA GAAGCGACCC CTGCGGGAGA GGCACCGGAC CCCTCCCCAA 1200
TGGCATCTCG CCTACGTGGG AGACGGGAGC CCCCTGTGGC CGACTCCACT ACCTCGCAGG 1260
CATTCCCCCT CCGCGCAGGA GGAAACGGAC AGCTTCAATA CTGGCCGTTC TCCTCTTCTG 1320
ACCTTTACAA CTGGAAAAAT AATAACCCTT CTTTTTCTGA AGATCCAGGT AAACTGACAG 1380
CTCTGATCGA GTCTGTTCTC ATCACCCATC AGCCCACCTG GGACGACTGT CAGCAGCTGT 1440
TGGGGACTCT GCTGACCGGA GAAGAAAAAC AACGGGTGCT CTTAGAGGCT AGAAAGGCGG 1500
TGCGGGGCGA TGATGGGCGC CCCACTCAAC TGCCCAATGA AGTCGATGCC GCTTTTCCCC 1560
TCGAGCGCCC AGACTGGGAT TACACCACCC AGGCAGGTAG GAACCACCTA GTCCACTATC 1620
GCCAGTTGCT CCTAGCGGGT CTCCAAAACG CGGGCAGAAG CCCCACCAAT TTGGCCAAGG 1680
TAAAAGGAAT AACACAAGGG CCCAATGAGT CTCCCTCGGC CTTCCTAGAG AGACTTAAGG 1740

AAGCCTATCG CAGGTACACT CCTTATGACC CTGAGGACCC AGGGCAAGAA ACTAATGTGT 1800
CTATGTCTTT CATTTGGCAG TCTGCCCCAG ACATTGGGAG AAAGTTAGAG AGGTTAGAAG 1860
ATTTAAAAAA CAAGACGCTT GGAGATTTGG TTAGAGAGGC AGAAAAGATC TTTAATAAAC 1920
GAGAAACCCC GGAAGAAAGA GAGGAACGTA TCAGGAGAGA AACAGAGGAA AAAGAAGAAC 1980
GCCGTAGGAC AGAGGATGAG CAGAAAGAGA AAGAAAGAGA TCGTAGGAGA CATAGAGAGA 2040
TGAGCAAGCT ATTGGCCACT GTCGTTAGTG GACAGAAACA GGATAGACAG GGAGGAGAAC 2100
GAAGGAGGTC CCAACTCGAT CGCGACCAGT GTGCCTACTG CAAAGAAAAG GGGCACTGGG 2160
CTAAAGATTG TCCCAAGAAA CCACGAGGAC CTCGGGGACC AAGACCCCAG ACCTCCCTCC 2220
TGACCCTAGA TGACTAGGGA GGTCAGGGTC AGGAGCCCCC CCCTGAACCC AGGATAACCC 2280
TCAAAGTCGG GGGGCAACCC GTCACCTTCC TGGTAGATAC TGGGGCCCAA CACTCCGTGC 2340
TGACCCAAAA TCCTGGACCC CTAAGTGATA AGTCTGCCTG GGTCCAAGGG GCTACTGGAG 2400
GAAAGCGGTA TCGCTGGACC ACGGATCGCA AAGTACATCT AGCTACCGGT AAGGTCACCC 2460
ACTCTTTCCT CCATGTACCA GACTGTCCCT ATCCTCTGTT AGGAAGAGAT TTGCTGACTA 2520
AACTAAAAGC CCAAATCCAC TTTGAGGGAT CAGGAGCTCA GGTTATGGGA CCAATGGGGC 2580
AGCCCCTGCA AGTGTTGACC CTAAATATAG AAGATGAGCA TCGGCTACAT GAGACCTCAA 2640
AAGAGCCAGA TGTTTCTCTA GGGTCCACAT GGCTGTCTGA TTTTCCTCAG GCCTGGGCGG 2700
AAACCGGGGG CATGGGACTG GCAGTTCGCC AAGCTCCTCT GATCATACCT CTGAAAGCAA 2760
CCTCTACCCC CGTGTCCATA AAACAATACC CCATGTCACA AGAAGCCAGA CTGGGGATCA 2820
AGCCCCACAT ACAGAGACTG TTGGACCAGG GAATACTGGT ACCCTGCCAG TCCCCCTGGA 2880
ACACGCCCCT GCTACCCGTT AAGAAACCAG GGACTAATGA TTATAGGCCT GTCCAGGATC 2940
TGAGAGAAGT CAACAAGCGG GTGGAAGACA TCCACCCCAC CGTGCCCAAC CCTTACAACC 3000
TCTTGAGCGG GCTCCCACCG TCCCACCAGT GGTACACTGT GCTTGATTTA AAGGATGCCT 3060
TTTTCTGCCT GAGACTCCAC CCCACCAGTC AGCCTCTCTT CGCCTTTGAG TGGAGAGATC 3120
CAGAGATGGG AATCTCAGGA CAATTGACCT GGACCAGACT CCCACAGGGT TTCAAAAACA 3180
GTCCCACCCT GTTTGATGAG GCACTGCACA GAGACCTAGC AGACTTCCGG ATCCAGCACC 3240
CAGACTTGAT CCTGCTACAG TACGTGGATG ACTTACTGCT GGCCGCCACT TCTGAGCTAG 3300
ACTGCCAACA AGGTACTCGG GCCCTGTTAC AAACCCTAGG GAACCTCGGG TATCGGGCCT 3360
CGGCCAAGAA AGCCCAAATT TGCCAGAAAC AGGTCAAGTA TCTGGGGTAT CTTCTAAAAG 3420

AGGGTCAGAG ATGGCTGACT GAGGCCAGAA AAGAGACTGT GATGGGGCAG CCTACTCCGA 3480

AGACCCCTCG ACAACTAAGG GAGTTCCTAG GGACGGCAGG CTTCTGTCGC CTCTGGATCC 3540

CTGGGTTTGC AGAAATGGCA GCCCCCTTGT ACCCTCTCAC CAAAACGGGG ACTCTGTTTA 3600

ATTGGGGCCC AGACCAACAA AAGGCCTATC AAGAAATCAA GCAAGCTCTT CTAACTGCCC 3660

CAGCCCTGGG GTTGCCAGAT TTGACTAAGC CCTTTGAACT CTTTGTCGAC GAGAAGCAGG 3720

GCTACGCCAA AGGTGTCCTA ACGCAAAAAC TGGGACCTTG GCGTCGGCCG GTGGCCTACC 3780

TGTCCAAAAA GCTAGACCCA GTAGCAGCTG GGTGGCCCCC TTGCCTACGG ATGGTAGCAG 3840

CCATTGCCGT ACTGACAAAG GATGCAGGCA AGCTAACCAT GGGACAGCCA CTAGTCATTC 3900

TGGCCCCCCA TGCAGTAGAG GCACTAGTCA AACAACCCCC CGACCGCTGG CTTTCCAACG 3960

CCCGGATGAC TCACTATCAG GCCTTGCTTT TGGACACGGA CCGGGTCCAG TTCGGACCGG 4020

TGGTAGCCCT GAACCCGGCT ACGCTGCTCC CACTGCCTGA GGAAGGGCTG CAACACAACT 4080

GCCTTGATAT CCTGGCCGAA GCCCACGGAA CCCGACCCGA CCTAACGGAC CAGCCGCTCC 4140

CAGACGCCGA CCACACCTGG TACACGGATG GAAGCAGTCT CTTACAAGAG GGACAGCGTA 4200

AGGCGGGAGC TGCGGTGACC ACCGAGACCG AGGTAATCTG GGCTAAAGCC CTGCCAGCCG 4260

GGACATCCGC TCAGCGGGCT GAACTGATAG CACTCACCCA GGCCCTAAAG ATGGCAGAAG 4320

GTAAGAAGCT AAATGTTTAT ACTGATAGCC GTTATGCTTT TGCTACTGCC CATATCCATG 4380

GAGAAATATA CAGAAGGCGT GGGTTGCTCA CATCAGAAGG CAAAGAGATC AAAAATAAAG 4440

ACGAGATCTT GGCCCTACTA AAAGCCCTCT TTCTGCCCAA AAGACTTAGC ATAATCCATT 4500

GTCCAGGACA TCAAAAGGGA CACAGCGCCG AGGCTAGAGG CAACCGGATG GCTGACCAAG 4560

CGGCCCGAAA GGCAGCCATC ACAGAGACTC CAGACACCTC TACCCTCCTC ATAGAAAATT 4620

CATCACCCTA CACCTCAGAA CATTTTCATT ACACAGTGAC TGATATAAAG GACCTAACCA 4680

AGTTGGGGGC CATTTATGAT AAAACAAAGA AGTATTGGGT CTACCAAGGA AAACCTGTGA 4740

TGCCTGACCA GTTTACTTTT GAATTATTAG ACTTTCTTCA TCAGCTGACT CACCTCAGCT 4800

TCTCAAAAAT GAAGGCTCTC CTAGAGAGAA GCCACAGTCC CTACTACATG CTGAACCGGG 4860

ATCGAACACT CAAAAATATC ACTGAGACCT GCAAAGCTTG TGCACAAGTC AACGCCAGCA 4920

AGTCTGCCGT TAAACAGGGA ACTAGGGTCC GCGGGCATCG GCCCGGCACT CATTGGGAGA 4980

TCGATTTCAC CGAGATAAAG CCCGGATTGT ATGGCTATAA ATATCTTCTA GTTTTTATAG 5040

ATACCTTTTC TGGCTGGATA GAAGCCTTCC CAACCAAGAA AGAAACCGCC AAGGTCGTAA 5100
CCAAGAAGCT ACTAGAGGAG ATCTTCCCCA GGTTCGGCAT GCCTCAGGTA TTGGGAACTG 5160
ACAATGGGCC TGCCTTCGTC TCCAAGGTGA GTCAGACAGT GGCCGATCTG TTGGGGATTG 5220
ATTGGAAATT ACATTGTGCA TACAGACCCC AAAGCTCAGG CCAGGTAGAA AGAATGAATA 5280
GAACCATCAA GGAGACTTTA ACTAAATTAA CGCTTGCAAC TGGCTCTAGA GACTGGGTGC 5340
TCCTACTCCC CTTAGCCCTG TACCGAGCCC GCAACACGCC GGGCCCCCAT GGCCTCACCC 5400
CATATGAGAT CTTATATGGG GCACCCCCGC CCCTTGTAAA CTTCCCTGAC CCTGACATGA 5460
CAAGAGTTAC TAACAGCCCC TCTCTCCAAG CTCACTTACA GGCTCTCTAC TTAGTCCAGC 5520
ACGAAGTCTG GAGACCTCTG GCGGCAGCCT ACCAAGAACA ACTGGACCGA CCGGTGGTAC 5580
CTCACCCTTA CCGAGTCGGC GACACAGTGT GGGTCCGCCG ACACCAGACT AAGAACCTAG 5640
AACCTCGCTG GAAAGGACCT TACACAGTCC TGCTGACCAC CCCCACCGCC CTCAAAGTAG 5700
ACGGCATCGC AGCTTGGATA CACGCCGCCC ACGTGAAGGC TGCCGACCCC GGGGGTGGAC 5760
CATCCTCTAG ACTGACATGG CGCGTTCAAC GCTCTCAAAA CCCCTTAAAA ATAAGGTTAA 5820
CCCGCGAGGC CCCCTAATCC CCTTAATTCT TCTGATGCTC AGAGGGGTCA GTACTGCTTC 5880
GCCCGGCTCC AGTCCTCATC AAGTCTATAA TATCACCTGG GAGGTAACCA ATGGAGATCG 5940
GGAGACGGTA TGGGCAACTT CTGGCAACCA CCCTCTGTGG ACCTGGTGGC CTGACCTTAC 6000
CCCAGATTTA TGTATGTTAG CCCACCATGG ACCATCTTAT TGGGGGCTAG AATATCAATC 6060
CCCTTTTTCT TCTCCCCCGG GGCCCCCTTG TTGCTCAGGG GGCAGCAGCC CAGGCTGTTC 6120
CAGAGACTGC GAAGAACCTT TAACCTCCCT CACCCCTCGG TGCAACACTG CCTGGAACAG 6180
ACTCAAGCTA GACCAGACAA CTCATAAATC AAATGAGGGA TTTTATGTTT GCCCCGGGCC 6240
CCACCGCCCC CGAGAATCCA AGTCATGTGG GGGTCCAGAC TCCTTCTACT GTGCCTATTG 6300
GGGCTGTGAG ACAACCGGTA GAGCTTACTG GAAGCCCTCC TCATCATGGG ATTTCATCAC 6360
AGTAAACAAC AATCTCACCT CTGACCAGGC TGTCCAGGTA TGCAAAGATA ATAAGTGGTG 6420
CAACCCCTTA GTTATTCGGT TTACAGACGC CGGGAGACGG GTTACTTCCT GGACCACAGG 6480
ACATTACTGG GGCTTACGTT TGTATGTCTC CGGACAAGAT CCAGGGCTTA CATTTGGGAT 6540
CCGACTCAGA TACCAAAATC TAGGACCCCG CGTCCCAATA GGGCCAAACC CCGTTCTGGC 6600
AGACCAACAG CCACTCTCCA AGCCCAAACC TGTTAAGTCG CCTTCAGTCA CCAAACCACC 6660
CAGTGGGACT CCTCTCTCCC CTACCCAACT TCCACCGGCG GGAACGGAAA ATAGGCTGCT 6720

AAACTTAGTA GACGGAGCCT ACCAAGCCCT CAACCTCACC AGTCCTGACA AAACCCAAGA 6780
GTGCTGGTTG TGTCTAGTAG CGGGACCCCC CTACTACGAA GGGGTTGCCG TCCTGGGTAC 6840
CTACTCCAAC CATACCTCTG CTCCAGCCAA CTGCTCCGTG GCCTCCCAAC ACAAGTTGAC 6900
CCTGTCCGAA GTGACCGGAC AGGGACTCTG CATAGGAGCA GTTCCCAAAA CACATCAGGC 6960
CCTATGTAAT ACCACCCAGA CAAGCAGTCG AGGGTCCTAT TATCTAGTTG CCCCTACAGG 7020
TACCATGTGG GCTTGTAGTA CCGGGCTTAC TCCATGCATC TCCACCACCA TACTGAACCT 7080
TACCACTGAT TATTGTGTTC TTGTCGAACT CTGGCCAAGA GTCACCTATC ATTCCCCCAG 7140
CTATGTTTAC GGCCTGTTTG AGAGATCCAA CCGACACAAA AGAGAACCGG TGTCGTTAAC 7200
CCTGGCCCTA TTATTGGGTG GACTAACCAT GGGGGGAATT GCCGCTGGAA TAGGAACAGG 7260
GACTACTGCT CTAATGGCCA CTCAGCAATT CCAGCAGCTC CAAGCCGCAG TACAGGATGA 7320
TCTCAGGGAG GTTGAAAAAT CAATCTCTAA CCTAGAAAAG TCTCTCACTT CCCTGTCTGA 7380
AGTTGTCCTA CAGAATCGAA GGGGCCTAGA CTTGTTATTT CTAAAAGAAG GAGGGCTGTG 7440
TGCTGCTCTA AAAGAAGAAT GTTGCTTCTA TGCGGACCAC ACAGGACTAG TGAGAGACAG 7500
CATGGCCAAA TTGAGAGAGA GGCTTAATCA GAGACAGAAA CTGTTTGAGT CAACTCAAGG 7560
ATGGTTTGAG GGACTGTTTA ACAGATCCCC TTGGTTTACC ACCTTGATAT CTACCATTAT 7620
GGGACCCCTC ATTGTACTCC TAATGATTTT GCTCTTCGGA CCCTGCATTC TTAATCGATT 7680
AGTCCAATTT GTTAAAGACA GGATATCAGT GGTCCAGGCT CTAGTTTTGA CTCAACAATA 7740
TCACCAGCTG AAGCCTATAG AGTACGAGCC ATAGATAAAA TAAAAGATTT TATTTAGTCT 7800
CCAGAAAAAG GGGGGAATGA AAGACCCCAC CTGTAGGTTT GGCAAGCTAG CTTAAGTAAC 7860
GCCATTTTGC AAGGCATGGA AAAATACATA ACTGAGAATA GAGAAGTTCA GATCAAGGTC 7920
AGGAACAGAT GGAACAGCTG AATATGGGCC AAACAGGATA TCTGTGGTAA GCAGTTCCTG 7980
CCCCGGCTCA GGGCCAAGAA CAGATGGAAC AGCTGAATAT GGGCCAAACA GGATATCTGT 8040
GGTAAGCAGT TCCTGCCCCG GCTCAGGGCC AAGAACAGAT GGTCCCCAGA TGCGGTCCAG 8100
CCCTCAGCAG TTTCTAGAGA ACCATCAGAT GTTTCCAGGG TGCCCCAAGG ACCTGAAATG 8160
ACCCTGTGCC TTATTTGAAC TAACCAATCA GTTCGCTTCT CGCTTCTGTT CGCGCGCTTC 8220
TGCTCCCCGA GCTCAATAAA AGAGCCCACA ACCCCTCACT CGGGGCGCCA GTCCTCCGAT 8280
TGACTGAGTC GCCCGGGTAC CCGTGTATCC AATAAACCCT CTTGCAGTTG CA 8332

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGTAACAGTC TGGCCCGAAT TCTCAGACAA ATACAG 36

2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CTGTATTTGT CTGAGAATTA AGGCTAGACT GTTACCAC 38

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATATATATAT ATCGATACCA TG 22

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGCGCCAAAC CTAAAC 16

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Ser Lys Asn Tyr Pro 5

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ACCATCCTCT GGACGGACAT G 21

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ACCCGGCCGT GGACGGACAT G 21

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 449 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 20..439

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
ATATATATAT ATCGATACC ATG GGG CAA ACC GTG ACT ACC CCT CTG TCC CTC        52
                     Met Gly Gln Thr Val Thr Thr Pro Leu Ser Leu
                       1               5                  10

ACA CTG GGC CAT TGG AAG GAC GTG GAA AGA ATT GCC CAT AAT CAA AGC       100
Thr Leu Gly His Trp Lys Asp Val Glu Arg Ile Ala His Asn Gln Ser
            15                  20                  25

GTG GAC TGC AAA AAA CGC AGG TGG GTG ACA TTT TGT AGC GCC GAG TGG       148
Val Asp Cys Lys Lys Arg Arg Trp Val Thr Phe Cys Ser Ala Glu Trp
        30                  35                  40

CCC ACA TTC AAT GTT GGC TGG CCT AGG GAT GGA ACT TTC AAT CGC GAT       196
Pro Thr Phe Asn Val Gly Trp Pro Arg Asp Gly Thr Phe Asn Arg Asp
    45                  50                  55

CTG ATT ACT CAA GTG AAA ATT AAA GTG TTC AGC CCC GGA CCC CAC GGC       244
Leu Ile Thr Gln Val Lys Ile Lys Val Phe Ser Pro Gly Pro His Gly
60                  65                  70                  75

CAT CCC GAT CAA GTT CCT TAT ATT GTC ACA TGG GAG GCT CTC GCT TTC       292
His Pro Asp Gln Val Pro Tyr Ile Val Thr Trp Glu Ala Leu Ala Phe
                80                  85                  90

GAT CCA CCA CCT TGG GTG AAA CCA TTC GTG CAT CCC AAA CCA CCT CCA       340
Asp Pro Pro Pro Trp Val Lys Pro Phe Val His Pro Lys Pro Pro Pro
            95                  100                 105

CCC CTC CCA CCC AGC GCT CCT AGC CTG CCC TTG GAG CCC CCA CGA AGC       388
Pro Leu Pro Pro Ser Ala Pro Ser Leu Pro Leu Glu Pro Pro Arg Ser
        110                 115                 120

ACA CCA CCC AGG AGC AGC TTG TAC CCT GCT CTG ACC CCC AGC CTC GGC       436
Thr Pro Pro Arg Ser Ser Leu Tyr Pro Ala Leu Thr Pro Ser Leu Gly
    125                 130                 135

GCC AAACCTAAAC                                                       449
Ala
140
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 140 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Met Gly Gln Thr Val Thr Thr Pro Leu Ser Leu Thr Leu Gly His Trp
 1               5                  10                  15

Lys Asp Val Glu Arg Ile Ala His Asn Gln Ser Val Asp Cys Lys Lys
            20                  25                  30

Arg Arg Trp Val Thr Phe Cys Ser Ala Glu Trp Pro Thr Phe Asn Val
        35                  40                  45

Gly Trp Pro Arg Asp Gly Thr Phe Asn Arg Asp Leu Ile Thr Gln Val
    50                  55                  60

Lys Ile Lys Val Phe Ser Pro Gly Pro His Gly His Pro Asp Gln Val
65                  70                  75                  80

Pro Tyr Ile Val Thr Trp Glu Ala Leu Ala Phe Asp Pro Pro Pro Trp
                85                  90                  95

Val Lys Pro Phe Val His Pro Lys Pro Pro Pro Pro Leu Pro Pro Ser
            100                 105                 110

Ala Pro Ser Leu Pro Leu Glu Pro Pro Arg Ser Thr Pro Pro Arg Ser
        115                 120                 125

Ser Leu Tyr Pro Ala Leu Thr Pro Ser Leu Gly Ala
    130                 135                 140
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 420 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..420

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
ATG GGC CAG ACT GTT ACC ACT CCC TTA AGT TTG ACC TTA GGT CAC TGG        48
Met Gly Gln Thr Val Thr Thr Pro Leu Ser Leu Thr Leu Gly His Trp
 1               5                  10                  15

AAA GAT GTC GAG CGG ATC GCT CAC AAC CAG TCG GTA GAT GTC AAG AAG        96
Lys Asp Val Glu Arg Ile Ala His Asn Gln Ser Val Asp Val Lys Lys
            20                  25                  30

AGA CGT TGG GTT ACC TTC TGC TCT GCA GAA TGG CCA ACC TTT AAC GTC       144
Arg Arg Trp Val Thr Phe Cys Ser Ala Glu Trp Pro Thr Phe Asn Val
        35                  40                  45

GGA TGG CCG CGA GAC GGC ACC TTT AAC CGA GAC CTC ATC ACC CAG GTT       192
Gly Trp Pro Arg Asp Gly Thr Phe Asn Arg Asp Leu Ile Thr Gln Val
    50                  55                  60

AAG ATC AAG GTC TTT TCA CCT GGC CCG CAT GGA CAC CCA GAC CAG GTC       240
Lys Ile Lys Val Phe Ser Pro Gly Pro His Gly His Pro Asp Gln Val
65                  70                  75                  80

CCC TAC ATC GTG ACC TGG GAA GCC TTG GCT TTT GAC CCC CCT CCC TGG       288
Pro Tyr Ile Val Thr Trp Glu Ala Leu Ala Phe Asp Pro Pro Pro Trp
                85                  90                  95

GTC AAG CCC TTT GTA CAC CCT AAG CCT CCG CCT CCT CTT CCT CCA TCC       336
Val Lys Pro Phe Val His Pro Lys Pro Pro Pro Pro Leu Pro Pro Ser
            100                 105                 110

GCC CCG TCT CTC CCC CTT GAA CCT CCT CGT TCG ACC CCG CCT CGA TCC       384
Ala Pro Ser Leu Pro Leu Glu Pro Pro Arg Ser Thr Pro Pro Arg Ser
        115                 120                 125

TCC CTT TAT CCA GCC CTC ACT CCT TCT CTA GGC GCC                       420
Ser Leu Tyr Pro Ala Leu Thr Pro Ser Leu Gly Ala
    130                 135                 140
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 140 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
Met Gly Gln Thr Val Thr Thr Pro Leu Ser Leu Thr Leu Gly His Trp
 1               5                  10                  15

Lys Asp Val Glu Arg Ile Ala His Asn Gln Ser Val Asp Val Lys Lys
            20                  25                  30

Arg Arg Trp Val Thr Phe Cys Ser Ala Glu Trp Pro Thr Phe Asn Val
```

```
        35                  40                  45

Gly Trp Pro Arg Asp Gly Thr Phe Asn Arg Asp Leu Ile Thr Gln Val
    50                  55                  60

Lys Ile Lys Val Phe Ser Pro Gly Pro His Gly His Pro Asp Gln Val
65                  70                  75                  80

Pro Tyr Ile Val Thr Trp Glu Ala Leu Ala Phe Asp Pro Pro Pro Trp
                85                  90                  95

Val Lys Pro Phe Val His Pro Lys Pro Pro Pro Pro Leu Pro Pro Ser
            100                 105                 110

Ala Pro Ser Leu Pro Leu Glu Pro Pro Arg Ser Thr Pro Pro Arg Ser
        115                 120                 125

Ser Leu Tyr Pro Ala Leu Thr Pro Ser Leu Gly Ala
    130                 135                 140
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2001 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
GGCCGACACC CAGAGTGGAC CATCCTCTGG ACGGACATGG CGCGTTCAAC GCTCTCAAAA      60

CCCCCTCAAG ATAAGATTAA CCCGTGGAAG CCCTTAATAG TCATGGGAGT CCTGTTAGGA     120

GTAGGGATGG CAGAGAGCCC CCATCAGGTC TTTAATGTAA CCTGGAGAGT CACCAACCTG     180

ATGACTGGGC GTACCGCCAA TGCCACCTCC CTCCTGGGAA CTGTACAAGA TGCCTTCCCA     240

AAATTATATT TTGATCTATG TGATCTGGTC GGAGAGGAGT GGGACCCTTC AGACCAGGAA     300

CCGTATGTCG GGTATGGCTG CAAGTACCCC GCAGGGAGAC AGCGGACCCG GACTTTTGAC     360

TTTTACGTGT GCCCTGGGCA TACCGTAAAG TCGGGGTGTG GGGGACCAGG AGAGGGCTAC     420

TGTGGTAAAT GGGGGTGTGA AACCACCGGA CAGGCTTACT GGAAGCCCAC ATCATCGTGG     480

GACCTAATCT CCCTTAAGCG CGGTAACACC CCCTGGGACA CGGGATGCTC TAAAGTTGCC     540

TGTGGCCCCT GCTACGACCT CTCCAAAGTA TCCAATTCCT TCCAAGGGGC TACTCGAGGG     600

GGCAGATGCA ACCCTCTAGT CCTAGAATTC ACTGATGCAG GAAAAAAGGC TAACTGGGAC     660
```

GGGCCCAAAT CGTGGGGACT GAGACTGTAC CGGACAGGAA CAGATCCTAT TACCATGTTC 720
TCCCTGACCC GGCAGGTCCT TAATGTGGGA CCCCGAGTCC CCATAGGGCC CAACCCAGTA 780
TTACCCGACC AAAGACTCCC TTCCTCACCA ATAGAGATTG TACCGGCTCC ACAGCCACCT 840
AGCCCCCTCA ATACCAGTTA CCCCCCTTCC ACTACCAGTA CACCCTCAAC CTCCCCTACA 900
AGTCCAAGTG TCCCACAGCC ACCCCCAGGA ACTGGAGATA GACTACTAGC TCTAGTCAAA 960
GGAGCCTATC AGGCGCTTAA CCTCACCAAT CCCGACAAGA CCCAAGAATG TTGGCTGTGC 1020
TTAGTGTCGG GACCTCCTTA TTACGAAGGA GTAGCGGTCG TGGGCACTTA TACCAATCAT 1080
TCCACCGCTC CGGCCAACTG TACGGCCACT TCCCAACATA AGCTTACCCT ATCTGAAGTG 1140
ACAGGACAGG GCCTATGCAT GGGGGCAGTA CCTAAAACTC ACCAGGCCTT ATGTAACACC 1200
ACCCAAAGCG CCGGCTCAGG ATCCTACTAC CTTGCAGCAC CCGCCGGAAC AATGTGGGCT 1260
TGCAGCACTG GATTGACTCC CTGCTTGTCC ACCACGGTGC TCAATCTAAC CACAGATTAT 1320
TGTGTATTAG TTGAACTCTG GCCCAGAGTA ATTTACCACT CCCCCGATTA TATGTATGGT 1380
CAGCTTGAAC AGCGTACCAA ATATAAAAGA GAGCCAGTAT CATTGACCCT GGCCCTTCTA 1440
CTAGGAGGAT TAACCATGGG AGGGATTGCA GCTGGAATAG GGACGGGGAC CACTGCCTTA 1500
ATTAAAACCC AGCAGTTTGA GCAGCTTCAT GCCGCTATCC AGACAGACCT CAACGAAGTC 1560
GAAAAGTCAA TTACCAACCT AGAAAAGTCA CTGACCTCGT TGTCTGAAGT AGTCCTACAG 1620
AACCGCAGAG GCCTAGATTT GCTATTCCTA AAGGAGGGAG GTCTCTGCGC AGCCCTAAAA 1680
GAAGAATGTT GTTTTTATGC AGACCACACG GGGCTAGTGA GAGACAGCAT GGCCAAATTA 1740
AGAGAAAGGC TTAATCAGAG ACAAAAACTA TTTGAGACAG GCCAAGGATG GTTCGAAGGG 1800
CTGTTTAATA GATCCCCCTG GTTTACCACC TTAATCTCCA CCATCATGGG ACCTCTAATA 1860
GTACTCTTAC TGATCTTACT CTTTGGACCT TGCATTCTCA ATCGATTGGT CCAATTTGTT 1920
AAAGACAGGA TCTCAGTGGT CCAGGCTCTG GTTTTGACTC AGCAATATCA CCAGCTAAAA 1980
CCCATAGAGT ACGAGCCATG A 2001

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CTAGCTAGCT AG 12

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 64 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

ATATATATAT ATCGATACCA TGGGGCAAAC CGTGACTACC CCTCTGTCCC TCACACTGGC 60

CCAA 64

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TTGATTATGG GCATTTCTTT CCACGTCCTT CCAATGGCCC AGTGTGAGGG A 51

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 72 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

AGAATTGCCC ATAATCAAAG CGTGGACGTC AAAAAACGCA GGTGGGTGAC ATTTTGTAGC 60

GCCGAGTGGC CC 72

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

AAGTTCCATC CCTAGGCCAG CCAACATTGA ATGTGGGCCA CTCGGCGCTA CA 52

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 71 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GGCCTAGGGA GGAACTTTCA ATCGCGATCT GATTACTCAA GTGAAAATTA AAGTGTTCAG 60

CCCCGGACCC C 71

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GTGACAATAT AAGGAACTTG ATCGGGATGG CCGTGGGGTC CGGGGCTGAA CA 52

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 72 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

AGTTCCTTAT ATTGTCACAT CGGAGGCTCT CGCTTTCGAT CCACCACCTT GGGTGAAACC 60

ATTCGTGCAT CC 72

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22

AGGAGCGCTG GGTGGGAGGG GTGGAGGTGG TTTGGGATGC ACGAATGGTT TC 52

(2) INFORMATION FOR SEQ ID NO:23

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 72 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CTCCCACCCA GCGCTCCTAG CCTGCCCTTG GAGCCCCCAC GAAGCACACC ACCCAGGAGC 60

AGCTTGTACC CT 72

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GTTTAGGTTT GGCGCCGAGG CTGGGGGTCA GAGCAGGGTA CAAGCTGCTC CT 52

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

ATATATATAT ATCGATACC 19

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GTTTAGGTTT GGCGCCGAGG 20

Claims

1. A retroviral vector construct comprising a 5' LTR, a tRNA binding site, a packaging signal, one or more heterologous sequences, an origin of second strand DNA synthesis and a 3' LTR, wherein said vector construct lacks *gag/pol* and *env* coding sequences.

2. The retroviral vector construct according to claim 1 wherein said vector construct lacks an extended packaging signal.

3. The retroviral vector construct according to claim 1 wherein said construct lacks a retroviral nucleic acid sequence upstream of said 5' LTR.

4. The retroviral vector construct according to claim 3 wherein said construct lacks an *env* coding sequence upstream of said 5' LTR.

5. The retroviral vector construct according to claim 1 wherein said construct lacks a retroviral packaging signal sequence downstream of said 3' LTR.

6. The retroviral vector construct according to any one of claims 1 to 5 wherein said retrovector is constructed from a retrovirus selected from the group consisting of amphotropic, ecotropic, xenotropic or polytropic viruses.

7. The retroviral vector construct according to any one of the claims 1 to 5 wherein said retrovector is constructed from a Murine Leukemia Virus.

8. The retroviral vector construct according to any one of claims 1 to 5, wherein said heterologous sequence is at least x kb in length, wherein x is selected from the group consisting of 2, 3, 4, 5, 6, 7 and 8.

9. The retroviral vector construct according to claim 8 wherein said heterologous sequence is a gene encoding a cytotoxic protein.

10. The retroviral vector construct according to claim 9 wherein said cytotoxic protein is selected from the group consisting of ricin, abrin, diphtheria toxin, cholera

toxin, gelonin, pokeweed, antiviral protein, tritin, Shigella toxin, and Pseudomonas exotoxin A.

11. The retroviral vector construct according to claim 8 wherein said heterologous sequence is an antisense sequence.

12. The retroviral vector construct according to claim 8 wherein said heterologous sequence encodes an immune accessory molecule.

13. The retroviral vector construct according to claim 12 wherein said immune accessory molecule is selected from the group consisting of IL-1, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, and IL-13.

14. The retroviral vector construct according to claim 12 wherein said immune accessory molecule is selected from the group consisting of IL-2, IL-12, IL-15 and gamma-interferon.

15. The retroviral vector construct according to claim 12 wherein said immune accessory molecule is selected from the group consisting of ICAM-1, ICAM-2, β-microglobin, LFA3, HLA class I and HLA class II molecules.

16. The retroviral vector construct according to claim 8 wherein said heterologous sequence encodes a gene product that activates a compound with little or no cytotoxicity into a toxic product.

17. The retroviral vector construct according to claim 16 wherein said gene product is selected from the group consisting of HSVTK and VZVTK.

18. The retroviral vector construct according to claim 8 wherein said heterologous sequence is a ribozyme.

19. The retroviral vector construct according to claim 8 wherein said heterologous sequence is a replacement gene.

20. The retroviral vector construct according to claim 19 wherein said replacement gene encodes a protein selected from the group consisting of Factor VIII, ADA, HPRT, CF and the LDL Receptor.

21. The retroviral vector construct according to claim 8 wherein said heterologous sequence encodes an immunogenic portion of a virus selected from the group consisting of HBV, HCV, HPV, EBV, FeLV, FIV, and HIV.

22. A *gag/pol* expression cassette, comprising a promoter operably linked to a *gag/pol* gene, and a polyadenylation sequence, wherein said *gag/pol* gene has been modified to contain codons which are degenerate for gag.

23. The *gag/pol* expression cassette according to claim 22 wherein the 5' terminal end of said *gag/pol* gene lacks a retroviral packaging signal sequence.

24. A *gag/pol* expression cassette, comprising a promoter operably linked to a *gag/pol* gene, and a polyadenylation sequence, wherein a 3' terminal end of said *gag/pol* gene has been deleted without affecting the biological activity of integrase.

25. The *gag/pol* expression cassette according to claim 24 wherein a 5' terminal end of said *gag/pol* gene has been modified to contain codons which are degenerate for gag.

26. The *gag/pol* expression cassette according to claim 24 wherein said *gag/pol* gene lacks a retroviral packaging signal sequence.

27. The *gag/pol* expression cassette according to claims 24 to 26 wherein said 3' terminal end has been deleted upstream of nucleotide 5751 of Sequence ID No. 1.

28. The *gag/pol* expression cassette according to any one of claims 22 to 26 wherein said promoter is a heterologous promoter.

29. The *gag/pol* expression cassette according to claim 28 wherein said promoter is selected from the group consisting of CMV IE, the HSVTK promoter, RSV promoter, Adenovirus major-later promoter and the SV40 promoter.

30. The *gag/pol* expression cassette according to any one of claims 22 to 26 wherein said polyadenylation sequence is a heterologous polyadenylation sequence.

31. The *gag/pol* expression cassette according to claim 30 wherein said heterologous polyadenylation sequence is selected from the group consisting of the SV40 late poly A signal and the SV40 early poly A signal.

32. A *gag/pol* expression cassette, comprising a promoter operably linked to a *gag/pol* gene, and a polyadenylation sequence, wherein said expression cassette does not co-encapsidate with a replication competent virus.

33. An *env* expression cassette, comprising a promoter operably linked to an *env* gene, and a polyadenylation sequence, wherein no more than 6 consecutive retroviral nucleotides are included upstream of said *env* gene.

34. An *env* expression cassette, comprising a promoter operably linked to an *env* gene, and a polyadenylation sequence, wherein said *env* expression cassette does not contain a consecutive sequence of more than 8 nucleotides which are found in a *gag/pol* gene.

35. An *env* expression cassette, comprising a promoter operably linked to an *env* gene, and a polyadenylation sequence, wherein a 3' terminal end of said *env* gene has been deleted without effecting the biological activity of env.

36. The *env* expression cassette according to claim 35 wherein said 3' terminal end of said gene has been deleted such that a complete R peptide is not produced by said expression cassette.

37. The *env* expression cassette according to claim 36 wherein said *env* gene is derived from a type C retrovirus, and wherein the 3' terminal end has been deleted such that said *env* gene includes less than 18 nucleic acids which encode said R peptide.

38. The *env* expression cassette according to claim 36 wherein said 3' terminal end has been deleted downstream from nucleotide 7748 of Sequence ID. No. 1.

39. The *env* expression cassette according to any one of claims 33 to 38 wherein said promoter is a heterologous promoter.

40. The *env* expression cassette according to claim 39 wherein said promoter is selected from the group consisting of CMV IE, the HSVTK promoter, RSV promoter, Adenovirus major-later promoter and the SV40 promoter.

41. The *env* expression cassette according to any one of claims 33 to 38 wherein said polyadenylation sequence is a heterologous polyadenylation sequence.

42. The *env* expression cassette according to claim 41 wherein said heterologous polyadenylation is selected from the group consisting of the SV40 late poly A signal and the SV40 early poly A signal.

43. A packaging cell line, comprising a *gag/pol* expression cassette and an *env* expression cassette, wherein said *gag/pol* expression cassette lacks a consecutive sequence of greater than 8 consecutive nucleotides which are found in said *env* expression cassette.

44. A packaging cell line, comprising a *gag/pol* expression cassette according to claims 22 to 32, and an *env* expression cassette.

46. A packaging cell line, comprising a *gag/pol* expression cassette, and an *env* expression cassette according to claims 33 to 42.

46. A producer cell line, comprising a packaging cell line according to any one of claims 43 to 45, and a retroviral vector construct.

47. The producer cell line according to claim 46 wherein said retroviral vector construct is a retroviral vector construct according to any one of claims 1 to 21.

48. A producer cell line, comprising a *gag/pol* expression cassette, an *env* expression cassette, and a retroviral vector construct, wherein said *gag/pol* expression cassette, *env* expression cassette and retroviral vector construct lack a consecutive sequence of greater than 8 nucleotides in common.

49. A method of producing a packaging cell, comprising:

(a) introducing a *gag/pol* expression cassette according to claims 22 to 32 into an animal cell;

(b) selecting a cell containing a *gag/pol* expression cassette which expresses high levels of *gag/pol*;

(c) introducing an *env* expression cassette into said selected cell; and

(d) selecting a cell which expresses high levels of *env*, and thereby producing said packaging cell.

50. A method of producing a packaging cell, comprising:

(a) introducing an *env* expression cassette according to claims 33 to 42 into an animal cell;

(b) selecting a cell which expresses high levels of *env*;

(c) introducing a *gag/pol* expression cassette into said selected cell; and

(d) selecting a cell containing a *gag/pol* expression cassette which expresses high levels of *gag/pol*, and thereby producing said packaging cell.

51. A method of producing recombinant retroviral particles, comprising introducing a retroviral vector construct into packaging cell line according to claim 49 or 50.

52. The method according claim 51 wherein said retroviral vector construct is a retroviral vector construct according to any one of claims 1 to 21.

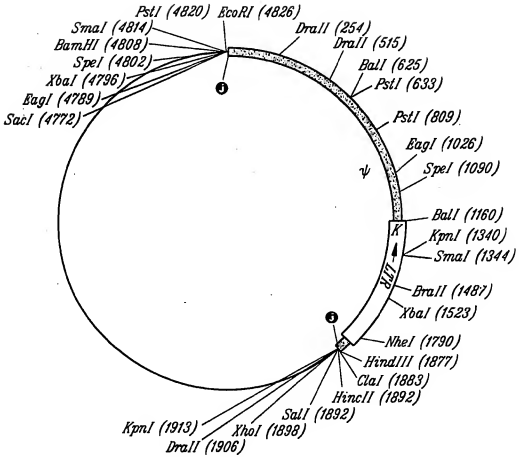

Fig. 1

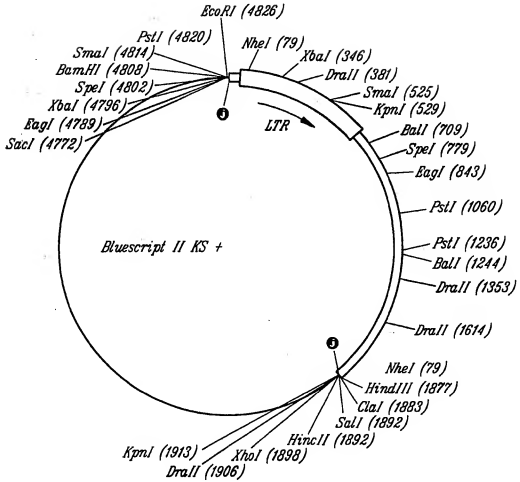

Fig. 2

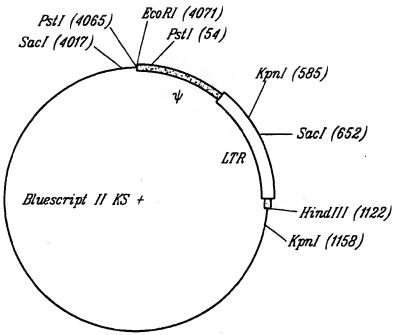

Fig. 3

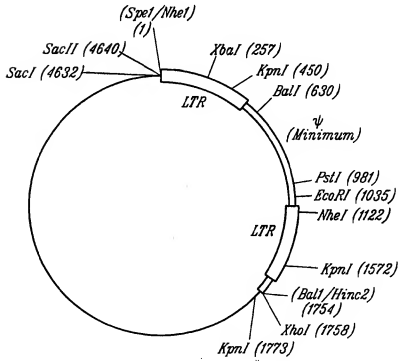

Fig. 4

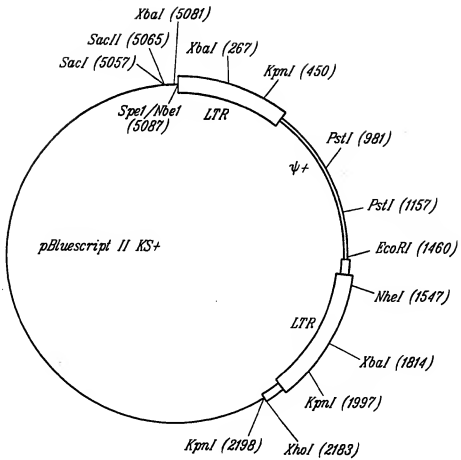

Fig. 5

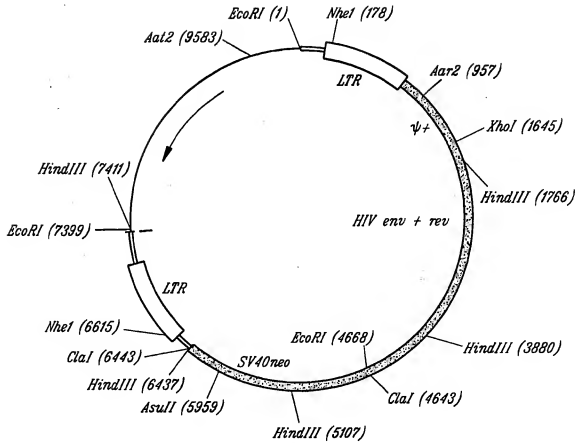

Fig. 6

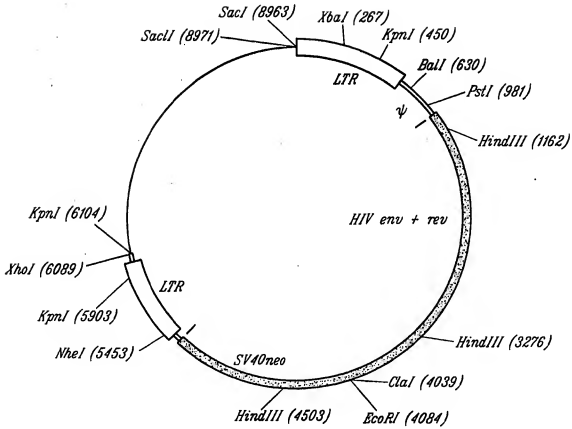

Fig. 7

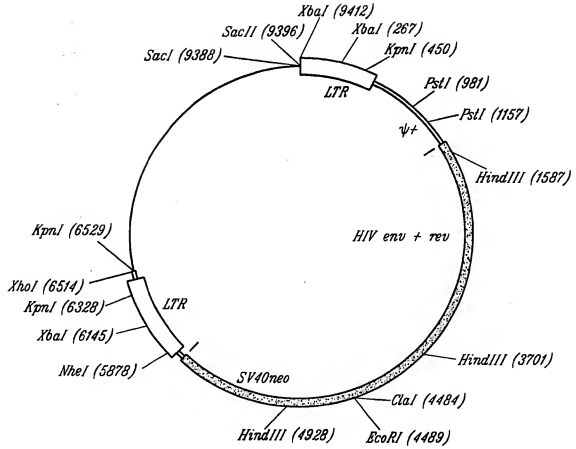

Fig. 8

```
   1 ATG GGC CAG ACT GTT ACC ACT CCC TTA AGT TTG ACC TTA GGT CAC TGG AAA
   1▶Met Gly Gln Thr Val Thr Thr Pro Leu Ser Leu Thr Leu Gly His Trp Lys

  52 GAT GTC GAG CGG ATC GCT CAC AAC CAG TCG GTA GAT GTC AAG AAG AGA CGT
  18▶Asp Val Glu Arg Ile Ala His Asn Gln Ser Val Asp Val Lys Lys Arg Arg

 103 TGG GTT ACC TTC TGC TCT GCA GAA TGG CCA ACC TTT AAC GTC GGA TGG CCG
  35▶Trp Val Thr Phe Cys Ser Ala Glu Trp Pro Thr Phe Asn Val Gly Trp Pro

 154 CGA GAC GGC ACC TTT AAC CGA GAC CTC ATC ACC CAG GTT AAG ATC AAG GTC
  52▶Arg Asp Gly Thr Phe Asn Arg Asp Leu Ile Thr Gln Val Lys Ile Lys Val

 205 TTT TCA CCT GGC CCG CAT GGA CAC CCA GAC CAG GTC CCC TAC ATC GTG ACC
  69▶Phe Ser Pro Gly Pro His Gly His Pro Asp Gln Val Pro Tyr Ile Val Thr

 256 TGG GAA GCC TTG GCT TTT GAC CCC CCT CCC TGG GTC AAG CCC TTT GTA CAC
  86▶Trp Glu Ala Leu Ala Phe Asp Pro Pro Pro Trp Val Lys Pro Phe Val His

 307 CCT AAG CCT CCG CCT CCT CTT CCT CCA TCC GCC CCG TCT CTC CCC CTT GAA
 103▶Pro Lys Pro Pro Pro Pro Leu Pro Pro Ser Ala Pro Ser Leu Pro Leu Glu

 358 CCT CCT CGT TCG ACC CCG CCT CGA TCC TCC CTT TAT CCA GCC CTC ACT CCT
 120▶Pro Pro Arg Ser Thr Pro Pro Arg Ser Ser Leu Tyr Pro Ala Leu Thr Pro

                    NarI (415)
 409 TCT CTA GGC GCC
 137▶Ser Leu Gly Ala
```

Fig. 9

```
A TAT ATA TAT ATC GAT ACC  ATG GGG CAA ACC GTG ACT ACC CCT CTG TCC
                        ▶Met Gly Gln Thr Val Thr Thr Pro Leu Ser

 CTC ACA CTG GGC CAT TGG AAG GAC GTG GAA AGA ATT GCC CAT AAT CAA AGC
▶Leu Thr Leu Gly His Trp Lys Asp Val Glu Arg Ile Ala His Asn Gln Ser

 GTG GAC GTC AAA AAA CGC AGG TGG GTG ACA TTT TGT AGC GCC GAG TGG CCC
▶Val Asp Val Lys Lys Arg Arg Trp Val Thr Phe Cys Ser Ala Glu Trp Pro

 ACA TTC AAT GTT GGC TGG CCT AGG GAT GGA ACT TTC AAT CGC GAT CTG ATT
▶Thr Phe Asn Val Gly Trp Pro Arg Asp Gly Thr Phe Asn Arg Asp Leu Ile

 ACT CAA GTG AAA ATT AAA GTG TTC AGC CCC GGA CCC CAC GGC CAT CCC GAT
▶Thr Gln Val Lys Ile Lys Val Phe Ser Pro Gly Pro His Gly His Pro Asp

 CAA GTT CCT TAT ATT GTC ACA TGG GAG GCT CTC GCT TTC GAT CCA CCA CCT
▶Gln Val Pro Tyr Ile Val Thr Trp Glu Ala Leu Ala Phe Asp Pro Pro Pro

 TGG GTG AAA CCA TTC GTG CAT CCC AAA CCA CCT CCA CCC CTC CCA CCC AGC
▶Trp Val Lys Pro Phe Val His Pro Lys Pro Pro Pro Pro Leu Pro Pro Ser

 GCT CCT AGC CTG CCC TTG GAG CCC CCA CGA AGC ACA CCA CCC AGG AGC AGC
▶Ala Pro Ser Leu Pro Leu Glu Pro Pro Arg Ser Thr Pro Pro Arg Ser Ser

                                        NarI
 TTG TAC CCT GCT CTG ACC CCC AGC CTC GGC GCC AAA    CCT AAA C
▶Leu Tyr Pro Ala Leu Thr Pro Ser Leu Gly Ala Lys ? ????????
```

Fig. 10

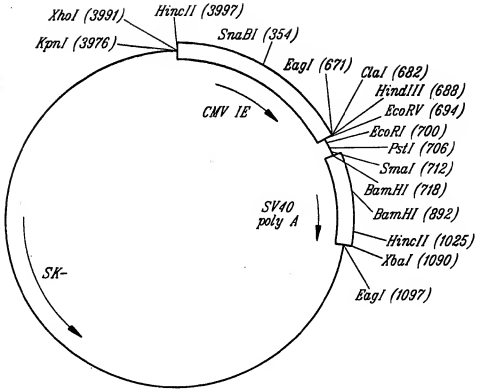

Fig. 11

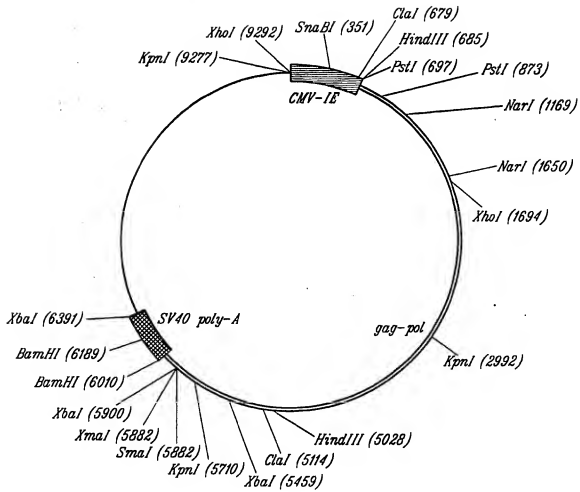

Fig. 12

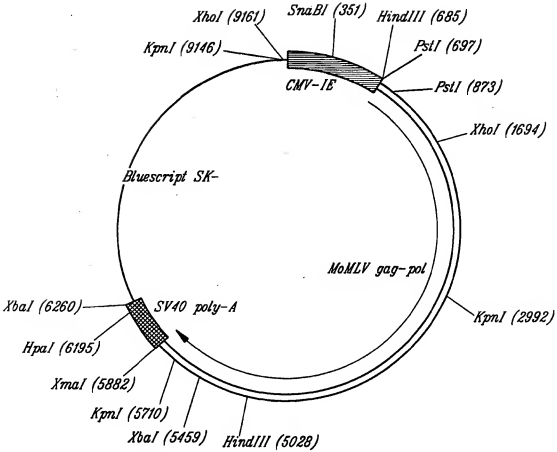

Fig. 13

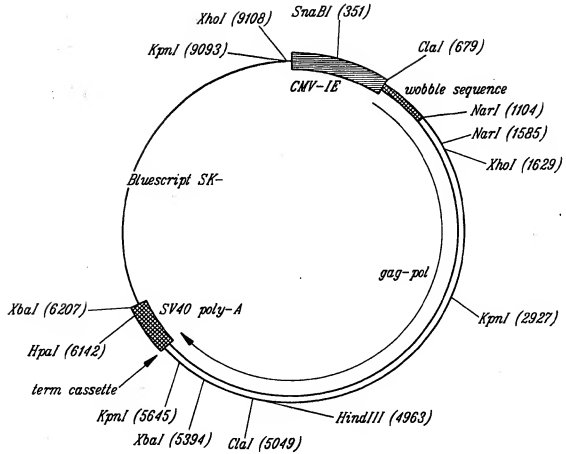

Fig. 14

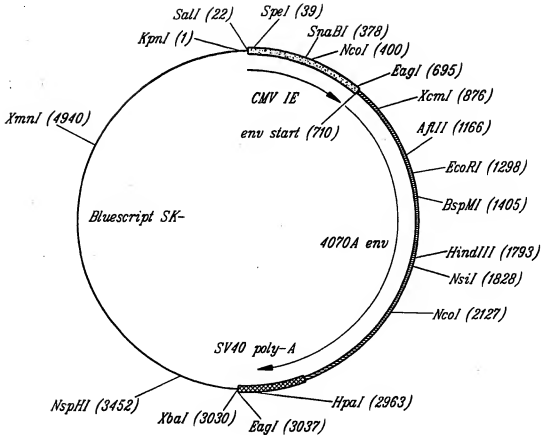

Fig. 15

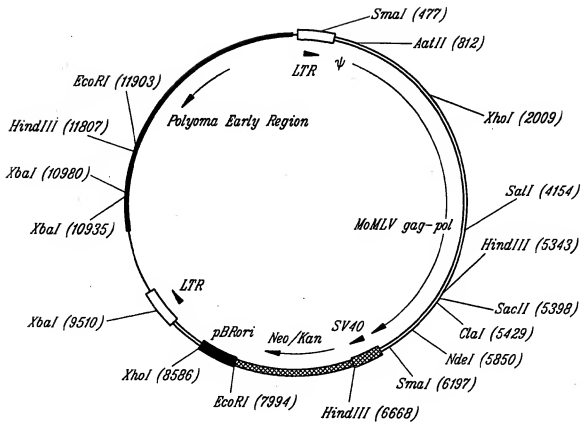

Fig. 16

Fig. 17A

VIRUS	SPECIES OF ISOLATION	TYPE[1]
AEV (Avian erthroblastosis virus)	chicken	C,X,T
ALV (avian leukosis virus)	chicken	C,N or X,N
AMV (avian myeloblastosis virus)	chicken	C,X,T
ASV (avian sarcoma virus)	chicken	C,X,T
BaEV (baboon endogenous virus)	baboon (*Papio ssp.*)	C,N,N
B1LN	*P. hamadryas*	
M7	*P. cynocephalus*	
M28	*P. cynocephalus*	
PP-1-Lu	*P. papio*	
TG-1-K	gelada	
BLV (bovine leukemia virus)	cow	C,X,N
BSV (bovine syncytial virus)	cow	S,X,N
CAEV (caprine arthritis-encephalitis virus)	goat	L,X,N
CERV-CI, CERV C-II	*Mus cervicolor*	C,N,N
CCC	cat	C,N,N
CPC-1	colobus monkey	C,N,N
CSRV (corn snake retrovirus)	corn snake	C,
CSV (chick syncytial virus)	chicken	C,X,N
DIAV (duck infectious anemia virus)	duck	C,X,N
DKV (deer kidney virus	black-tailed deer	C,N,N
DPC-1	agouti	C,N,N
EIAV (equine infectious anemia virus)	horse	C,X,N
ESV (Esh sarcoma virus)	chicken	C,X,T
FeLV (feline leukemia virus)	cat	C,N or X,N
FeSV (feline sarcoma virus)	cat	C,X,T
GA (Gardner-Arnstein)		
SM (McDonough)		
ST (Snyder-Theilen)		
FS-1	*Felis sylvestris* (wildcat)	C,N,N
FSFV (feline syncytium-forming virus	cat	S,X,N
FuSV (Fujinami sarcoma virus)	chicken	C,X,T
GALV (gibbon ape leukemia virus)	gibbon	C,X,N
GLV (goat leukoencephalitis virus)	see CAEV	
GPV (golden pheasant virus)	golden pheasant	C,N,N
HaLV (hamster leukemia virus)	hamster	C,N,N
IVL (induced leukemia virus)	chicken	C,N,N
LLV (lymphoid leukosis virus)	see ALV	
LPDV (lymphoproliferative disease of turkeys	turkey	C,X,T
M432	*Mus cervicolor*	B,N,N
M832	*Mus caroli*	B,N,N

[1] The first letter denotes classification: (B) B-type oncovirus; (C) C-type oncovirus; (D) D-type oncovirus; (L) lentivirus; (S) spumavirus. The second letter denotes origin: (N) enogenous; (X) exogenous; (R) recombinant. The third letter denotes ability to indice morphological transformation: (T) transforming (i.e., containing an *onc* sequence); (N) nontransforming; (?) unknown.

MAC-1	stumptail monkey	C,N,N
Maedi	sheep	L,X,N
MAV (myeloblastosis-associated virus)	chicken	C,X,N
MC29 (myelocytomatosis virus)	chicken	C,X,T
MCF (mink cell focus-inducing virus)	mouse	C,NR,N
MH2 (myelocytomatosis virus)	chicken	C,X,T
MiLV (mink leukemia virus)	mink	C,N,N
MLV (murine leukemia virus)	mouse	C,X or N,N
Ab (Abelson)		C,X,T
Fr (Friend)		C,X,N
Graffi		C,X,N
Gross		C,N,N
Ki (Kirsten)		C,X,N
Mo (Moloney)		C,X,N
Ra (Rauscher)		C,X,N
MMC-1	rhesus monkey	C,N,N
MMTV (mouse mammary tumor virus)	mouse	B,X or N,N
MPMV (Mason-Pfizer monkey virus)	rhesus monkey	D,X,N
MSV (murine sarcoma virus)	mouse	C,X,T
BALB		
FBJ (Finkel-Biskis-Jinkins)		
FBR		
Gz (Gazdar)		
Ha (Harvey)		
Ki (Kirsten)		
Mo (Moloney)		
MPV[1] (myeloproliferative)		
OS2 (osteosarcoma)		
MyLV (myeloid leukemia)	mouse	C,X,N
OK10 (myelocytomatosis virus)	chicken	C,X,T
OMC-1	owl monkey	C,N,N
PK-15	pig	C,N,N
PO-1-Lu	langur	D,N,N
PPV (progressive pneumonia virus)	sheep	L,X,N
PRCII, PRCIV (Poultry Research Centre)	chicken	C,X,T
R-35	rat	C,X?,T
RaLV (rat leukemia virus)	rat	C,X,N
RaSV (rat sarcoma virus)	rat	C,X,T
RAV-*n* (Rous-associated virus)	see ALV	
RAV-0 (Rous-associated virus 0)	chicken	C,N,N
RAV-60 (Rous-associated virus 60)	chicken	C,R,N
RAV-61 (Rous-associated virus 61)	ring-necked pheasant	C,R,N
RD114	cat	C,N,N
REAV (reticuloendotheliosis-associated virus)	turkey	C,X,N

Fig. 17B

REV (reticuloendotheliosis virus)	birds	C,X,N
REV-T (reticuloendotheliosis virus-transforming	turkey	C,X,T
RIF (Rous interference factor)	see ALV	
RPL-*n* (Regional Poultry Laboratory)	see ALV	
RPV (ring-necked pheasant virus)	ring-necked pheasant	C,R,N
RSV (Rous sarcoma virus)	chicken	C,X,T
B77 (Bratislava)		
BH (Bryan high titer)		
BS (Bryan standard)		
CZ (Carr-Zilber)		
EH (Engelbreth-Holm)		
HA (Harris)		
PR (Prague)		
SR (Schmidt-Ruppin)		
SFV-*n* (simian foamy virus)	monkey	S,X,N
SFFV (spleen focus-forming virus)	mouse	C,X, or R,N or T
Friend		
MPV		
Rauscher		
SiSV (simian sarcoma virus)	see SSV	
SLV (simian lymphoma virus)	see GALV	
SMRV (squirrel monkey retrovirus)	squirrel monkey	D,N,N
SMV (simian myelogenous leukemia virus)	see GALV	
SSAV (simian sarcoma-associated virus)	woolly monkey	C,X,N
SSV (simian sarcoma virus)	woolly monkey	C,X,T
TRV-1	tree shrew	C,N,N
UR-*n* (University of Rochester)	chicken	C,X,T
Vand C-I	tree mouse	C,N,N
Visna	sheep	L,X,N
VRV (viper retrovirus)	Russell's viper	C,N,?
WMV (woolly monkey virus)	see SSV	
WoLV (woolly monkey leukemia virus)	see SSAV	
Y73 (Yamaguchi 73)	chicken	C,X,T

Fig. 17C

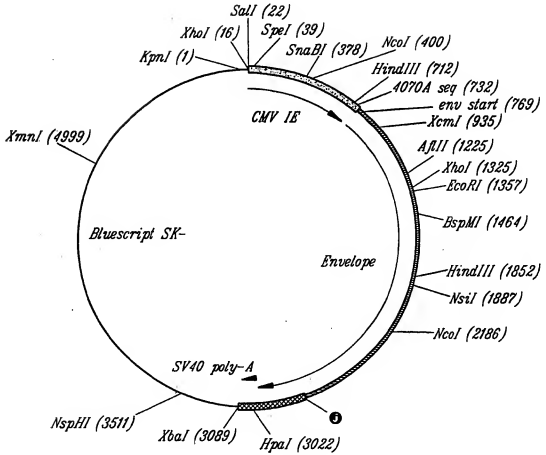

Fig. 18

CMV Promoter – wobble gag – SVneo – LTR

Fig. 19A

CMV Promoter – normal gag – SVneo – LTR

Fig. 19B

CORRECTED VERSION*

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/86, 5/10, 7/04, 9/00, C07K 14/00, 14/005, 14/15, 14/54

A3

(11) International Publication Number: WO 95/30763

(43) International Publication Date: 16 November 1995 (16.11.95)

(21) International Application Number: PCT/US95/05789

(22) International Filing Date: 9 May 1995 (09.05.95)

(30) Priority Data:
08/240,030 9 May 1994 (09.05.94) US

(71) Applicant: VIAGENE, INC. [US/US]; 11055 Roselle Street, San Diego, CA 92121 (US).

(72) Inventor: RESPESS, James, G.; 4966 Lamont Street, San Diego, CA 92109 (US).

(74) Agents: McMASTERS, David, D. et al.; Seed and Berry, 6300 Columbia Center, 701 Fifth Avenue, Seattle, WA 98104-7092 (US).

(81) Designated States: AM, AU, BB, BG, BR, BY, CA, CN, CZ, EE, ES, FI, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LV, MD, MG, MN, MW, MX, NO, NZ, PL, RO, RU, SD, SG, SI, SK, TJ, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(88) Date of publication of the international search report:
4 April 1996 (04.04.1996)

(54) Title: RETROVIRAL VECTORS HAVING A REDUCED RECOMBINATION RATE

```
      A TAT ATA TAT ATC GAT ACC  ATG GGG CAA ACC GTG ACT ACC CCT CTG TCC
                               ▸Met Gly Gln Thr Val Thr Thr Pro Leu Ser

  CTC ACA CTG GGC CAT TGG AAG GAC GTG GAA AGA ATT GCC CAT AAT CAA AGC
▸Leu Thr Leu Gly His Trp Lys Asp Val Glu Arg Ile Ala His Asn Gln Ser

  GTG GAC GTC AAA AAA CGC AGG TGG GTG ACA TTT TGT AGC GCC GAG TGG CCC
▸Val Asp Val Lys Lys Arg Arg Trp Val Thr Phe Cys Ser Ala Glu Trp Pro

  ACA TTC AAT GTT GGC TGG CCT AGG GAT GGA ACT TTC AAT CGC GAT CTG ATT
▸Thr Phe Asn Val Gly Trp Pro Arg Asp Gly Thr Phe Asn Arg Asp Leu Ile

  ACT CAA GTG AAA ATT AAA GTG TTC AGC CCC GGA CCC CAC GGC CAT CCC GAT
▸Thr Gln Val Lys Ile Lys Val Phe Ser Pro Gly Pro His Gly His Pro Asp

  CAA GTT CCT TAT ATT GTC ACA TGG GAG GCT CTC GCT TTC GAT CCA CCA CCT
▸Gln Val Pro Tyr Ile Val Thr Trp Glu Ala Leu Ala Phe Asp Pro Pro Pro

  TGG GTG AAA CCA TTC GTG CAT CCC AAA CCA CCT CCA CCC CTC CCA CCC AGC
▸Trp Val Lys Pro Phe Val His Pro Lys Pro Pro Pro Pro Leu Pro Pro Ser

  GCT CCT AGC CTG CCC TTG GAG CCC CCA CGA AGC ACA CCA CCC AGG AGC AGC
▸Ala Pro Ser Leu Pro Leu Glu Pro Pro Arg Ser Thr Pro Pro Arg Ser Ser

                                        NarI
  TTG TAC CCT GCT CTG ACC CCC AGC CTC GGC GCC AAA   CCT AAA C
▸Leu Tyr Pro Ala Leu Thr Pro Ser Leu Gly Ala Lys ? ????????
```

(57) Abstract

Retroviral vector constructs are described which have a 5' LTR, a tRNA binding site, a packaging signal, one or more heterologous sequences, an origin of second strand synthesis and a 3' LTR, wherein the vector construct lacks retroviral *gag/pol* or *env* coding sequences. In addition, *gag/pol*, and *env* expression cassettes are described wherein the expression cassettes lack a consecutive sequence of more than 8 nucleotides in common. The above-described retroviral vector constructs, *gag/pol* and *env* expression cassettes may be utilized to construct producer cell lines which preclude the formation of replication competent virus.

* (Referred to in PCT Gazette No. 03/1996, Section II)

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo			SE	Sweden
CH	Switzerland	KR	Republic of Korea	SI	Slovenia
CI	Côte d'Ivoire	KZ	Kazakhstan	SK	Slovakia
CM	Cameroon	LI	Liechtenstein	SN	Senegal
CN	China	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
CZ	Czech Republic	LV	Latvia	TJ	Tajikistan
DE	Germany	MC	Monaco	TT	Trinidad and Tobago
DK	Denmark	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	US	United States of America
FI	Finland	ML	Mali	UZ	Uzbekistan
FR	France	MN	Mongolia	VN	Viet Nam
GA	Gabon				

INTERNATIONAL SEARCH REPORT

Int 'ional Application No
PCT/US 95/05789

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/86 C12N5/10 C12N7/04 C12N9/00 C07K14/00
C07K14/005 C07K14/15 C07K14/54

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J.VIROLOGY, vol. 66, no. 3, March 1992 page 1571-1578 T. TCHENIO AND T. HEIDMANN 'High-frequency intracellular transposition of a defective mammalian provirus detected by an In Situ colorimetric assay' see the whole document ---	1,6-8
P,X	WO,A,94 29438 (CELL GENESYS, INC.) 22 December 1994 see page 5, line 11 - page 7, line 35 see page 9, line 5 - page 12, line 32 see page 14, line 16 - page 21, line 22 --- -/--	22-26, 28-37, 39-46, 48-51

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 January 1996	2 9. 02. 96
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Donath, C

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	J.VIROLOGY, vol. 68, no. 6, June 1994 pages 3888-3895, C. PAROLIN ET AL. 'Analysis in human immunodeficiency virus type 1 vectors of cis-acting sequences that affect gene transfer into human lymphocytes' see the whole document ---	1,6
Y	WO,A,89 07150 (THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK) 10 August 1989 see the whole document ---	1-52
Y	VIROLOGY, vol. 167, 1988 pages 400-406, D. MARKOWITZ ET AL. 'Construction and use of a safe and efficient amphotropic packaging cell line' see the whole document ---	1-52
Y	PROC.NATL.ACAD.SCI. USA, vol. 85, 1988 pages 6460-6464, O. DANOS AND R.C. MULLIGAN 'Safe and efficient generation of recombinant retroviruses with amphotropic and ecotropic host ranges' see the whole document ---	1-52
Y	WO,A,90 02806 (WHITEHEAD INSTITUTE FOR BIOMEDICAL RESEARCH) 22 March 1990 see the whole document ---	1-52
Y	INT.CONF.AIDS (NETHERLANDS), JULY 19-24 1992, vol. 8, no. 2, 1992 page PA82, Abstract No. POA 2477 V. NEUBAUER ET AL. 'Construction of a retroviral vector for intracellular immunization with a HIV antisense sequence' see the whole document ---	11
A	PROC.NATL.ACAD.SCI. USA, vol. 84, 1987 pages 156-160, J. PRICE ET AL. 'Lineage analysis in the vertebrate nervous system by retrovirus-mediated gene transfer' see the whole document -----	

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int[illegible]tional Application No
PCT/US 95/05789

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9429438	22-12-94	AU-B-	7246294	03-01-95
WO-A-8907150	10-08-89	AU-B-	3069189	25-08-89
		CA-A-	1315719	06-04-93
		US-A-	5278056	11-01-94
WO-A-9002806	22-03-90	EP-A-	0432216	19-06-91
		JP-T-	4500158	16-01-92
		US-A-	5449614	12-09-95

Form PCT/ISA/210 (patent family annex) (July 1992)

CORRECTED VERSION*

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/86, 5/10, 7/04, 9/00, C07K 14/00, 14/005, 14/15, 14/54

A2

(11) International Publication Number: WO 95/30763

(43) International Publication Date: 16 November 1995 (16.11.95)

(21) International Application Number: PCT/US95/05789

(22) International Filing Date: 9 May 1995 (09.05.95)

(30) Priority Data:
08/240,030 9 May 1994 (09.05.94) US

(71) Applicant: VIAGENE, INC. [US/US]; 11055 Roselle Street, San Diego, CA 92121 (US).

(72) Inventor: RESPESS, James, G.; 4966 Lamont Street, San Diego, CA 92109 (US).

(74) Agents: McMASTERS, David, D. et al.; Seed and Berry, 6300 Columbia Center, 701 Fifth Avenue, Seattle, WA 98104-7092 (US).

(81) Designated States: AM, AU, BB, BG, BR, BY, CA, CN, CZ, EE, ES, FI, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LV, MD, MG, MN, MW, MX, NO, NZ, PL, RO, RU, SD, SG, SI, SK, TJ, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: RETROVIRAL VECTORS HAVING A REDUCED RECOMBINATION RATE

```
A TAT ATA TAT ATC GAT ACC  ATG GGG CAA ACC GTG ACT ACC CCT CTG TCC
                         ▶Met Gly Gln Thr Val Thr Thr Pro Leu Ser

CTC ACA CTG GGC CAT TGG AAG GAC GTG GAA AGA ATT GCC CAT AAT CAA AGC
▶Leu Thr Leu Gly His Trp Lys Asp Val Glu Arg Ile Ala His Asn Gln Ser

GTG GAC GTC AAA AAA CGC AGG TGG GTG ACA TTT TGT AGC GCC GAG TGG CCC
▶Val Asp Val Lys Lys Arg Arg Trp Val Thr Phe Cys Ser Ala Glu Trp Pro

ACA TTC AAT GTT GGC TGG CCT AGG GAT GGA ACT TTC AAT CGC GAT CTG ATT
▶Thr Phe Asn Val Gly Trp Pro Arg Asp Gly Thr Phe Asn Arg Asp Leu Ile

ACT CAA GTG AAA ATT AAA GTG TTC AGC CCC GGA CCC CAC GGC CAT CCC GAT
▶Thr Gln Val Lys Ile Lys Val Phe Ser Pro Gly Pro His Gly His Pro Asp

CAA GTT CCT TAT ATT GTC ACA TGG GAG GCT CTC GCT TTC GAT CCA CCA CCT
▶Gln Val Pro Tyr Ile Val Thr Trp Glu Ala Leu Ala Phe Asp Pro Pro Pro

TGG GTG AAA CCA TTC GTG CAT CCC AAA CCA CCT CCA CCC CTC CCA CCC AGC
▶Trp Val Lys Pro Phe Val His Pro Lys Pro Pro Pro Pro Leu Pro Pro Ser

GCT CCT AGC CTG CCC TTG GAG CCC CCA CGA AGC ACA CCA CCC AGG AGC AGC
▶Ala Pro Ser Leu Pro Leu Glu Pro Pro Arg Ser Thr Pro Pro Arg Ser Ser

                                        NarI
TTG TAC CCT GCT CTG ACC CCC AGC CTC GGC GCC AAA   CCT AAA C
▶Leu Tyr Pro Ala Leu Thr Pro Ser Leu Gly Ala Lys ? ????????
```

(57) Abstract

Retroviral vector constructs are described which have a 5' LTR, a tRNA binding site, a packaging signal, one or more heterologous sequences, an origin of second strand synthesis and a 3' LTR, wherein the vector construct lacks retroviral *gag/pol* or *env* coding sequences. In addition, *gag/pol*, and *env* expression cassettes are described wherein the expression cassettes lack a consecutive sequence of more than 8 nucleotides in common. The above-described retroviral vector constructs, *gag/pol* and *env* expression cassettes may be utilized to construct producer cell lines which preclude the formation of replication competent virus.

* (Referred to in PCT Gazette No. 03/1996, Section II)

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgyzstan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

Description

RETROVIRAL VECTORS HAVING A REDUCED RECOMBINATION RATE

Technical Field

The present invention relates generally to retroviral vectors for use in gene transfer, and more specifically, to retroviral vectors which are constructed such that the formation of replication competent virus by recombination is precluded.

Background of the Invention

Retroviruses are RNA viruses which can replicate and integrate into a host cell's genome through a DNA intermediate. This DNA intermediate, or provirus, may be stably integrated into the host's cellular DNA. Retroviruses are known to be responsible for a wide variety of diseases in both man and animals, including for example AIDS and a wide variety of cancers.

Although retroviruses can cause disease, they also have a number of properties that lead them to be considered as one of the most promising techniques for genetic therapy of disease. These properties include: (1) efficient entry of genetic material (the vector genome) into cells; (2) an active efficient process of entry into the target cell nucleus; (3) relatively high levels of gene expression; (4) minimal pathological effects on target cells; and (5) the potential to target particular cellular subtypes through control of the vector-target cell binding and tissue-specific control of gene expression. In using a retrovirus for genetic therapy, a foreign gene of interest may be incorporated into the retrovirus in place of normal retroviral RNA. When the retrovirus injects its RNA into a cell, the foreign gene is also introduced into the cell, and may then be integrated into the host's cellular DNA as if it were the retrovirus itself. Expression of this foreign gene within the host results in expression of foreign protein by the host cell.

Most retroviral vector systems which have been developed for gene therapy are based on murine retroviruses. Briefly, these retroviruses exist in two forms, as proviruses integrated into a host's cellular DNA, or as free virions. The virion form of the virus contains the structural and enzymatic proteins of the retrovirus (including reverse transcriptase), two RNA copies of the viral genome, and portions of the cell's plasma membrane in which is embedded the viral envelope glycoprotein. The genome is organized into four main regions: the Long Terminal Repeat (LTR), and the *gag*, *pol*, and *env* genes. The LTR may be found at both ends of the proviral genome, is a composite of the 5' and 3' ends of the RNA genome, and contains *cis*-acting elements

necessary for the initiation and termination of transcription. The three genes *gag, pol,* and *env* are located between the terminal LTRs. The *gag* and *pol* genes encode, respectively, internal viral structures and enzymatic proteins (such as integrase). The *env* gene encodes the envelope glycoprotein (designated gp70 and p15e) which confers infectivity and host range specificity of the virus, as well as the "R" peptide of undetermined function.

An important consideration in using retroviruses for gene therapy is the availability of "safe" retroviruses. Packaging cell lines and vector producing cell lines have been developed to meet this concern. Briefly, this methodology employs the use of two components, a retroviral vector and a packaging cell line (PCL). The retroviral vector contains long terminal repeats (LTRs), the foreign DNA to be transferred and a packaging sequence (ψ). This retroviral vector will not reproduce by itself because the genes which encode structural and envelope proteins are not included within the vector genome. The PCL contains genes encoding the *gag, pol,* and *env* proteins, but does not contain the packaging signal "ψ". Thus, a PCL can only form empty virion particles by itself. Within this general method, the retroviral vector is introduced into the PCL, thereby creating a vector-producing cell line (VCL). This VCL manufactures virion particles containing only the retroviral vector's (foreign) genome, and therefore has previously been considered to be a safe retrovirus vector for therapeutic use.

There are, however, several shortcomings with the current use of VCLs. One issue involves the generation of "live virus" (*i.e.*, replication competent retrovirus; RCR) by the VCL. Briefly, RCR can be produced in conventional producer cells when: (1) The vector genome and the helper genomes recombine with each other; (2) The vector genome or helper genome recombines with homologous cryptic endogenous retroviral elements in the producer cell; or (3) Cryptic endogenous retroviral elements reactivate (*e.g.*, xenotropic retroviruses in mouse cells).

Another issue is the propensity of mouse based VCLs to package endogenous retrovirus-like elements (which can contain oncogenic gene sequences) at efficiencies close to that with which they package the desired retroviral vector. Such elements, because of their retrovirus-like structure, are transmitted to the target cell to be treated at frequencies that parallel its transfer of the desired retroviral vector sequence.

A third issue is the ability to make sufficient retroviral vector particles at a suitable concentration to: (1) treat a large number of cells (*e.g.*, 10^8 - 10^{10}); and (2) manufacture vector particles at a commercially viable cost.

In order to construct safer PCLs, researchers have generated deletions of the 5' LTR and portions of the 3' LTR of helper elements (*see,* Miller and Buttimore, *Mol. Cell. Biol. 6*:2895-2902, 1986). When such cells are used, two recombination events are necessary to form the wild-type, replication competent genome. Nevertheless, results from several laboratories have indicated that even when several deletions are present, RCR may still be generated (*see*, Bosselman et al., *Mol. Cell. Biol. 7*:1797-1806, 1987; Danos and Mulligan, *Proc. Nat'l. Acad. Sci. USA 81*:6460-6464, 1988). In addition, cell lines containing both 5' and 3' LTR deletions which have been constructed have thus far not proven useful since they produce relatively low titers (Dougherty et al., *J. Virol. 63*:3209-3212, 1989).

One of the more recent approaches to constructing safer packaging cell lines involves the use of complementary portions of helper virus elements, divided among two separate plasmids, one containing *gag* and *pol*, and the other containing *env* (*see,* Markowitz et al., *J. Virol. 62*:1120-1124; and Markowitz et al., *Virology 167*:600-606, 1988. One benefit of this double-plasmid system is that three recombination events are required to generate a replication competent genome. Nevertheless, these double-plasmid vectors have also suffered from the drawback of including portions of the retroviral LTRs, and therefore remain capable of producing infectious virus.

The present invention overcomes the difficulties of recombination and low titer associated with many of the prior packaging cell lines, and further provides other related advantages.

Summary of the Invention

Briefly stated, the present invention provides compositions and methods for the construction of packaging cell lines which preclude the formation of RCR by homologous recombination. Within one aspect of the invention, recombinant retroviral vector constructs (RETROVECTOR™) are provided comprising a 5' LTR, a tRNA binding site, a packaging signal, one or more heterologous sequences, an origin of second strand DNA synthesis, and a 3' LTR, wherein the retroviral vector construct lacks *gag/pol* and *env* coding sequences. Within one embodiment of the invention, the retroviral vector construct lacks an extended packaging signal. Within one embodiment, the retroviral vector construct lacks a retroviral nucleic acid sequence upstream of the 5' LTR. Within a preferred embodiment, the retroviral vector constructs lack an *env* coding sequence upstream of the 5' LTR. Retroviral vector constructs of the present invention may be constructed from one or more retroviruses, including, for example, a

wide variety of amphotropic, ecotropic, xenotropic, and polytropic viruses (*see e.g.*, Figures 17A, B, and C).

As noted above, retroviral vector constructs of the present invention include one or more heterologous sequences. Within certain embodiments of the invention, the heterologous sequence is at least x kb in length, wherein x is selected from the group consisting of 1, 2, 3, 4, 5, 6, 7 and 8. Within one embodiment, the heterologous sequence is a gene encoding a cytotoxic protein, such as, for example, ricin, abrin, diphtheria toxin, cholera toxin, gelonin, pokeweed, antiviral protein, tritin, Shigella toxin, and Pseudomonas exotoxin A. Within other embodiments the heterologous sequence may be an antisense sequence, or an immune accessory molecule. Representative examples of immune accessory molecules include IL-1, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-13, and IL-14. Particularly preferred immune accessory molecules may be selected from the group consisting of IL-2, IL-12, IL-15 and gamma-interferon, or the group consisting of ICAM-1, ICAM-2, β-microglobin, LFA3, HLA class I and HLA class II molecules.

Within other embodiments of the invention, the heterologous sequence may encode a gene product that activates a compound with little or no cytotoxicity into a toxic product. Representative examples of such gene products include type I thymidine kinases such as HSVTK and VZVTK. Within another embodiment, the heterologous sequence may be a ribozyme. Within yet other embodiments, the heterologous sequence is a replacement gene, which encode proteins such as Factor VIII, ADA, HPRT, CF and the LDL Receptor. Within other embodiments, the heterologous sequence encodes an immunogenic portion of a virus selected from the group consisting of HBV, HCV, HPV, EBV, FeLV, FIV, and HIV.

Within other aspects of the present invention, *gag/pol* expression cassettes are provided, comprising a promoter operably linked to a *gag/pol* gene, and a polyadenylation sequence, wherein the *gag/pol* gene has been modified to contain codons which are degenerate for gag. Within one embodiment, the 5' terminal end of the *gag/pol* gene lacks a retroviral packaging signal sequence. Within other aspects *gag/pol* expression cassettes are provided comprising a promoter operably linked to a *gag/pol* gene, and a polyadenylation sequence, wherein the expression cassette does not co-encapsidate with a replication competent virus.

Within another aspect of the present invention, *gag/pol* expression cassettes are provided comprising a promoter operably linked to a *gag/pol* gene, and a polyadenylation sequence, wherein a 3' terminal end of the *gag/pol* gene has been deleted without effecting the biological activity of integrase. Within one embodiment, a

5' terminal end of the *gag/pol* gene has been modified to contain codons which are degenerate for gag. Within a further embodiment, the 5' terminal end of the *gag/pol* gene lacks a retroviral packaging signal sequence. Within other embodiments, the 3' terminal end has been deleted upstream (5') of nucleotide 5751 of SEQ ID NO: 1.

Within other aspects of the present invention, *env* expression cassettes are provided, comprising a promoter operably linked to an *env* gene, and a polyadenylation sequence, wherein no more than 6 retroviral nucleotides are included upstream of the *env* gene. Within another aspect, *env* expression cassettes are provided comprising a promoter operably linked to an *env* gene, and a polyadenylation sequence, wherein the *env* expression cassette does not contain a consecutive sequence of more than 8 nucleotides which are found in a *gag/pol* gene. Within yet another aspect, *env* expression cassettes are provided comprising a promoter operably linked to an *env* gene, and a polyadenylation sequence, wherein a 3' terminal end of the *env* gene has been deleted without effecting the biological activity of env. Within one embodiment, the 3' terminal end of the gene has been deleted such that a complete R peptide is not produced by the expression cassette. Within a further embodiment, the *env* gene is derived from a type C retrovirus, and the 3' terminal end has been deleted such that the *env* gene includes less than 18 nucleic acids which encode the R peptide. Within a preferred embodiment, the 3' terminal end has been deleted downstream from nucleotide 7748 of SEQ ID NO: 1.

Within various embodiments of the invention, the promoters of the *gag/pol* and *env* expression cassettes described above are heterologous promoters, such as CMV IE, the HVTK promoter, RSV promoter, Adenovirus major-later promoter and the SV40 promoter. Within other embodiments, the polyadenylation sequence is a heterologous polyadenylation sequence, such as the SV40 late poly A Signal and the SV40 early poly A Signal.

Within another aspect of the present invention, packaging cell lines are provided, comprising a *gag/pol* expression cassette and an *env* expression cassette, wherein the *gag/pol* expression cassette lacks a consecutive sequence of greater than 20, preferably greater than 15, more preferably greater than 10, and most preferably greater than 8 consecutive nucleotides which are found in the *env* expression cassette. Within other aspects, producer cell lines are provided comprising a *gag/pol* expression cassette, *env* expression cassette, and a retroviral vector construct, wherein the *gag/pol* expression cassette, *env* expression cassette and retroviral vector construct lack a consecutive sequence of greater than 20, preferably greater than 15, more preferably greater than 10, and most preferably greater than 8 nucleotides in common.

Representative examples of such retroviral vector constructs, *gag/pol* and *env* expression cassettes are described in more detail below.

Within yet another aspect of the present invention, producer cell lines are provided comprising a packaging cell line as described above, and a retroviral vector construct. Within another aspect of the present invention, producer cell lines are provided comprising a *gag/pol* expression cassette, *env* expression cassette and a retroviral vector construct, wherein the *gag/pol* expression cassette, *env* expression cassette and retroviral vector construct lack a consecutive sequence of greater than eight nucleotides in common.

Within other aspects of the invention, methods of producing a packaging cell line are provided, comprising the steps of (a) introducing a *gag/pol* expression cassette as described above into an animal cell; (b) selecting a cell containing a *gag/pol* expression cassette which expresses high levels of *gag/pol*, (c) introducing an *env* expression cassette into said selected cell, and (d) selecting a cell which expresses high levels of *env* and thereby producing the packaging cell. Within other aspects of the invention, the *env* expression cassette may be introduced into the cell first, followed by the *gag/pol* expression cassette. Within other aspects, methods are provided for producing recombinant retroviral particles comprising the step of introducing a retroviral vector construct into a packaging cell as described above. Within preferred embodiments, the retroviral vector construct is one of the retroviral vector constructs described above.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references are set forth below which describe in more detail certain procedures or compositions (*e.g.*, plasmids, etc.), and are therefore incorporated by reference in their entirety.

Brief Description of the Drawings

Figure 1 is a schematic illustration of pKS2+Eco57I-LTR(+).

Figure 2 is a schematic illustration of pKS2+Eco57I-LTR(-).

Figure 3 is a schematic illustration of pKS2+LTR-EcoRI.

Figure 4 is a schematic illustration of pR1.

Figure 5 is a schematic illustration of pR2.

Figure 6 is a schematic illustration of pKT1.

Figure 7 is a schematic illustration of pRI-HIVenv.

Figure 8 is a schematic illustration of pR2-HIVenv.

Figure 9 is a representative "prewobble" sequence for a MoMLV *gag/pol* (*see also* SEQ I.D. Nos. 11 and 12).

Figure 10 is a representative "wobble" sequence for a MoMLV *gag/pol* (*see also* SEQ. I.D. Nos. 9 and 10).

Figure 11 is a schematic illustration of pHCMV-PA.

Figure 12 is a schematic illustration of pCMV *gag/pol*.

Figure 13 is a schematic illustration of pCMVgpSma.

Figure 14 is a schematic illustration of pCMVgp-X.

Figure 15 is a schematic illustration of pCMV env-X.

Figure 16 is a schematic illustration of pRgpNeo.

Figures 17A, B and C comprise a table which sets forth a variety of retroviruses which may be utilized to construct the retroviral vector constructs, *gag/pol* expression cassettes and *env* expression cassettes of the present invention.

Figure 18 is a schematic illustration of pCMV Envam-Eag-X-less.

Figure 19A is a diagrammatic illustration of a "wobble" *-gag* construct. Figure 19B is a diagrammatic illustration of a "normal" *-gag* construct.

Detailed Description of the Invention

Prior to setting forth the invention, it may be helpful to an understanding thereof to first set forth definitions of certain terms that will be used hereinafter.

"Retroviral vector construct" refers to an assembly which is, within preferred embodiments of the invention, capable of directing the expression of a sequence(s) or gene(s) of interest. Briefly, the retroviral vector construct must include a 5' LTR, a tRNA binding site, a packaging signal, one or more heterologus sequences, an origin of second strand DNA synthesis and a 3' LTR. A wide variety of heterologous sequences may be included within the vector construct, including for example, sequences which encode a protein (*e.g.*, cytotoxic protein, disease-associated antigen, immune accessory molecule, or replacement gene), or which are useful as a molecule itself (*e.g.*, as a ribozyme or antisense sequence). Alternatively, the heterologous sequence may merely be a "stuffer" or "filler" sequence, which is of a size sufficient to allow production of viral particles containing the RNA genome. Preferably, the heterologous sequence is at least 1, 2, 3, 4, 5, 6, 7 or 8 kB in length.

The retroviral vector construct may also include transcriptional promoter/enhancer or locus defining element(s), or other elements which control gene expression by means such as alternate splicing, nuclear RNA export, post-translational modification of messenger, or post-transcriptional modification of protein. Optionally,

the retroviral vector construct may also include selectable markers such as Neo, TK, hygromycin, phleomycin, histidinol, or DHFR, as well as one or more specific restriction sites and a translation termination sequence.

"Expression cassette" refers to an assembly which is capable of directing the expression of the sequence(s) or gene(s) of interest. The expression cassette must include a promoter which, when transcribed, is operably linked to the sequence(s) or gene(s) of interest, as well as a polyadenylation sequence. Within preferred embodiments of the invention, both the promoter and the polyadenylation sequence are from a source which is heterologous to the helper elements (*i.e.*, *gag/pol* and *env*). Expression cassettes of the present invention may be utilized to express a *gag/pol* gene or an *env* gene. In addition, the expression cassettes may also be utilized to express one or more heterologous sequences either from a *gag/pol* and/or *env* expression cassette, or from a entirely different expression cassette.

Within preferred embodiments of the invention, the expression cassettes described herein may be contained within a plasmid construct. In addition to the components of the expression cassette, the plasmid construct may also include a bacterial origin of replication, one or more selectable markers, a signal which allows the plasmid construct to exist as single-stranded DNA (*e.g.*, a M13 origin of replication), a multiple cloning site, and a "mammalian" origin of replication (*e.g.*, a SV40 or adenovirus origin of replication).

PREPARATION OF RETROVIRAL VECTOR CONSTRUCTS, *GAG/POL* EXPRESSION CASSETTES AND *ENV* EXPRESSION CASSETTES

As noted above, the present invention provides compositions and methods for constructing packaging cells which preclude the formation of replication competent virus by homologous recombination. The following sections describe the preparation of retroviral vector constructs, *gag/pol* expression cassettes, and *env* expression cassettes.

1. *Construction of retroviral vector constructs*

Within one aspect of the present invention, retroviral vector constructs are provided comprising a 5' LTR, a tRNA binding site, a packaging signal, one or more heterologous sequences, an origin of second strand DNA synthesis and a 3' LTR, wherein the vector construct lacks *gag/pol* or *env* coding sequences. Briefly, Long Terminal Repeats ("LTRs") are subdivided into three elements, designated U5, R and U3. These elements contain a variety of signals which are responsible for the biological

activity of a retrovirus, including for example, promoter and enhancer elements which are located within U3. LTR's may be readily identified in the provirus due to their precise duplication at either end of the genome.

The tRNA binding site and origin of second strand DNA synthesis are also important for a retrovirus to be biologically active, and may be readily identified by one of skill in the art. For example, tRNA binds to a retroviral tRNA binding site by Watson-Crick base pairing, and is carried with the retrovirus genome into a viral particle. The tRNA is then utilized as a primer for DNA synthesis by reverse transcriptase. The tRNA binding site may be readily identified based upon its location just downstream from the 5' LTR. Similarly, the origin of second strand DNA synthesis is, as its name implies, important for the second strand DNA synthesis of a retrovirus. This region, which is also referred to as the poly-purine tract, is located just upstream of the 3' LTR.

In addition to 5' and 3' LTRs, a tRNA binding site, and an origin of second strand DNA synthesis, retroviral vector constructs of the present invention also comprise a packaging signal, as well as one or more heterologous sequences, each of which is discussed in more detail below.

Retroviral vector constructs of the present invention may be readily constructed from a wide variety of retroviruses, including for example, B, C, and D type retroviruses as well as spumaviruses and lentiviruses (*see* RNA Tumor Viruses, Second Edition, Cold Spring Harbor Laboratory, 1985). Briefly, viruses are often classified according to their morphology as seen under electron microscopy. Type "B" retroviruses appear to have an eccentric core, while type "C" retroviruses have a central core. Type "D" retroviruses have a morphology intermediate between type B and type C retroviruses. Representative examples of suitable retroviruses include those set forth below in Figures 17A, B and C (*see* RNA Tumor Viruses at pages 2-7), as well as a variety of xenotropic retroviruses (*e.g.*, NZB-X1, NZB-X2 and NZB_{9-1} (*see* O'Neill et al., *J. Vir. 53*:100-106, 1985)) and polytropic retroviruses (*e.g.*, MCF and MCF-MLV (*see* Kelly et al., *J. Vir. 45*(1):291-298, 1983)). Such retroviruses may be readily obtained from depositories or collections such as the American Type Culture Collection ("ATCC"; Rockville, Maryland), or isolated from known sources using commonly available techniques.

Particularly preferred retroviruses for the preparation or construction of retroviral vector constructs of the present invention include retroviruses selected from the group consisting of Avian Leukosis Virus, Bovine Leukemia Virus, Murine Leukemia Virus, Mink-Cell Focus-Inducing Virus, Murine Sarcoma Virus, Reticuloendotheliosis virus, Gibbon Ape Leukemia Virus, Mason Pfizer Monkey Virus,

and Rous Sarcoma Virus. Particularly preferred Murine Leukemia Viruses include 4070A and 1504A (Hartley and Rowe, *J. Virol. 19*:19-25, 1976), Abelson (ATCC No. VR-999), Friend (ATCC No. VR-245), Graffi, Gross (ATCC No. VR-590), Kirsten, Harvey Sarcoma Virus and Rauscher (ATCC No. VR-998), and Moloney Murine Leukemia Virus (ATCC No. VR-190). Particularly preferred Rous Sarcoma Viruses include Bratislava, Bryan high titer (*e.g.*, ATCC Nos. VR-334, VR-657, VR-726, VR-659, and VR-728), Bryan standard, Carr-Zilber, Engelbreth-Holm, Harris, Prague (*e.g.*, ATCC Nos. VR-772, and 45033), and Schmidt-Ruppin (*e.g.* ATCC Nos. VR-724, VR-725, VR-354).

Any of the above retroviruses may be readily utilized in order to assemble or construct retroviral vector constructs, packaging cells, or producer cells of the present invention given the disclosure provided herein, and standard recombinant techniques (*e.g.*, Sambrook et al, *Molecular Cloning: A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, 1989; Kunkle, *PNAS 82*:488, 1985). Further, within certain embodiments of the invention, portions of the retroviral vector construct may be derived from different retroviruses. For example, within one embodiment of the invention, retrovector LTRs may be derived from a Murine Sarcoma Virus, a tRNA binding site from a Rous Sarcoma Virus, a packaging signal from a Murine Leukemia Virus, and an origin of second strand synthesis from an Avian Leukosis Virus. Similarly, portions of a packaging cell line may be derived from different viruses (*e.g.*, a *gag/pol* expression cassette may be constructed from a Moloney Murine Leukemia Virus, and an *env* expression cassette from a Mason Pfizer Monkey virus).

As noted above, within various aspects of the present invention, retroviral vector constructs are provided which have packaging signals, and which lack both *gag/pol* and *env* coding sequences. As utilized within the context of the present invention, a packaging signal should be understood to refer to that sequence of nucleotides which is not required for synthesis, processing or translation of viral RNA or assembly of virions, but which is required in *cis* for encapsidation of genomic RNA (*see* Mann et al., *Cell 33*:153-159, 1983; RNA Tumor Viruses, Second Edition, *supra*). Further, as utilized herein, the phrase "lacks *gag/pol* or *env* coding sequences" should be understood to refer to retrovectors which contain less than 20, preferably less than 15, more preferably less than 10, and most preferably less than 8 consecutive nucleotides which are found in *gag/pol* or *env* genes, and in particular, within *gag/pol* or *env* expression cassettes that are used to construct packaging cell lines for the retroviral vector construct. Representative examples of such retroviral vector constructs are set forth in more detail below and in Example 1.

As an illustration, within one embodiment of the invention construction of retroviral vector constructs which lack *gag/pol* or *env* sequences may be accomplished by preparing retroviral vector constructs which lack an extended packaging signal. As utilized herein, the phrase "extended packaging signal" refers to a sequence of nucleotides beyond the minimum core sequence which is required for packaging, that allows increased viral titer due to enhanced packaging. As an example, for the Murine Leukemia Virus MoMLV, the minimum core packaging signal is encoded by the sequence (counting from the 5' LTR cap site) from approximately nucleotide 144 of SEQ. I.D. No. 1, up through the *Pst* I site (nucleotide 567 of SEQ. I.D. No. 1). The extended packaging signal of MoMLV includes the sequence beyond nucleotide 567 up through the start of the *gag/pol* gene (nucleotide 621), and beyond nucleotide 1040. Thus, within this embodiment retroviral vector constructs which lack extended packaging signal may be constructed from the MoMLV by deleting or truncating the packaging signal downstream of nucleotide 567.

Within other embodiments of the invention, retroviral vector constructs are provided wherein the packaging signal that extends into, or overlaps with, retroviral *gag/pol* sequence is deleted or truncated. For example, in the representative case of MoMLV, the packaging signal is deleted or truncated downstream of the start of the *gag/pol* gene (nucleotide 621 of SEQ ID NO: 1). Within preferred embodiments of the invention, the packaging signal is terminated at nucleotide 570, 575, 580, 585, 590, 595, 600, 610, 615 or 617 of SEQ ID NO: 1.

Within other aspects of the invention, retroviral vector constructs are provided which include a packaging signal that extends beyond the start of the *gag/pol* gene (*e.g.*, for MoMLV, beyond nucleotide 621 of SEQ ID NO: 1). When such retroviral vector constructs are utilized, it is preferable to utilize packaging cell lines for the production of recombinant viral particles wherein the 5' terminal end of the *gag/pol* gene in a *gag/pol* expression cassette has been modified to contain codons which are degenerate for *gag*. Such *gag/pol* expression cassettes are described in more detail below in section 2, and in Example 3.

Within other aspects of the present invention, retroviral vector constructs are provided comprising a 5' LTR, a tRNA binding site, a packaging signal, an origin of second strand DNA synthesis and a 3' LTR, wherein the retrovector plasmid construct does not contain a retroviral nucleic acid sequence upstream of the 5' LTR. As utilized within the context of the present invention, the phrase "does not contain a retroviral nucleic acid sequence upstream of the 5' LTR" should be understood to mean that the retrovector plasmid construct contains less than 20, preferably less than 15, more

preferably less than 10, and most preferably less than 8 consecutive nucleotides which are found in a retrovirus, and more specifically, in a retrovirus which is homologous to the retroviral vector construct, upstream of and/or contiguous with the 5' LTR. Within preferred embodiments, the retrovector plasmid constructs do not contain an *env* coding sequence (as discussed below) upstream of the 5' LTR. A particularly preferred embodiment of such retrovector plasmid constructs is set forth in more detail below in Example 1.

Within a further aspect of the present invention, retrovector plasmid constructs are provided comprising a 5' LTR, a tRNA binding site, a packaging signal, an origin of second strand DNA synthesis and a 3' LTR, wherein the retrovector plasmid construct does not contain a retroviral packaging signal sequence downstream of the 3' LTR. As utilized herein, the term "packaging signal sequence" should be understood to mean a sequence sufficient to allow packaging of the RNA genome. A representative example of such a retroviral vector construct is set forth in more detail below in Example 1.

2. *Construction of gag/pol expression cassettes*

As noted above, the present invention also provides a variety of *gag/pol* expression cassettes which, in combination with the retroviral vector constructs and *env* expression cassettes of the present invention, enable the construction of packaging cell lines and producer cell lines which preclude the formation of replication competent virus. Briefly, retroviral *gag/pol* genes contain a *gag* region which encodes a variety of structural proteins that make up the core matrix and nucleocapsid, and a *pol* region which contains genes which encode (1) a protease for the processing of *gag/pol* and *env* proteins, (2) a reverse transcriptase polymerase, (3) an RNase H, and (4) an integrase, which is necessary for integration of the retroviral provector into the host genome. Although retroviral *gag/pol* genes may be utilized to construct the *gag/pol* expression cassettes of the present invention, a variety of other non-retroviral (and non-viral) genes may also be utilized to construct the *gag/pol* expression cassette. For example, a gene which encodes retroviral RNase H may be replaced with genes which encode bacterial (*e.g.*, *E. coli* or *Thermus thermophilus*) RNase H. Similarly, a retroviral integrase gene may be replaced by other genes with similar function (*e.g.*, yeast retrotransposon TY3 integrase).

Within one aspect of the invention, *gag/pol* expression cassettes are provided comprising a promoter operably linked to a *gag/pol* gene, and a polyadenylation sequence, wherein the *gag/pol* gene has been modified to contain

codons which are degenerate for gag. Briefly, as noted above, in wild-type retrovirus the extended packaging signal of the retrovirus overlaps with sequences which encode gag and pol. Thus, in order to eliminate the potential of crossover between the retroviral vector construct and the *gag/pol* expression cassette, as well as to eliminate the possiblity of co-encapsidation of the *gag/pol* expression cassette and replication competent virus or retroviral vector constructs, sequences of overlap should be eliminated. Within one embodiment of the invention, elimination of such overlap is accomplished by modifying the *gag/pol* gene (and more specifically, regions which overlap with the retroviral vector construct, such as the extended packaging signal) to contain codons that are degenerate (*i.e.*, that "wobble") for gag. In particular, within preferred embodiments of the invention codons are selected which encode biologically active gag/pol protein (*i.e.*, capable of producing a competent retroviral particle, in combination with an *env* expressing element, and a RNA genome), and which lack any packaging signal sequence, including in particular, extended packaging signal sequence. As utilized herein, the phrase "lacks any retroviral packaging signal sequence" should be understood to mean that the *gag/pol* expression cassette contains less than 20, preferably less than 15, more preferably less than 10, and most preferably less than 8 consecutive nucleotides which are identical to a sequence found in a retroviral packaging signal (*e.g.*, in the case of MoMLV, extending up and through the *Xho* 1 site at approximately nucleotide number 1561). A particularly preferred example of such modified codons which are degenerate for gag is shown in Figure 10, and in Example 3, although the present invention should not be so limited. In particular, within other embodiments, at least 25, 50, 75, 100, 125 or 135 *gag* codons are modified or "wobbled" from the native *gag* sequence within the *gag/pol* expression cassettes of the present invention.

In addition to eliminating overlap between the retroviral vector construct and the *gag/pol* gene, it is also preferable to eliminate any potential overlap between the *gag/pol* gene and the *env* gene in order to prohibit the possibility of homologous recombination. This may be accomplished in at least two principal ways: (1) by deleting a portion of the *gag/pol* gene which encodes the integrase protein, and in particular, that portion of the gene which encodes the integrase protein which overlaps with the *env* coding sequence, or (2) by selecting codons which are degenerate for integrase and/or env.

Thus, within one aspect of the present invention *gag/pol* expression cassettes are provided comprising a promoter operably linked to a *gag/pol* gene, and a polyadenylation sequence or signal, wherein a 3' terminal end of the gene has been deleted without effecting the biological activity of the integrase. (The biological activity

of integrase may be readily determined by detection of an integration event, either by DNA analysis or by expression of a transduced gene; *see* Roth et al., *J. Vir.* *65*(4):2141-2145, 1991.) As an example, in the Murine Leukemia Virus MoMLV (SEQ ID. NO. 1), the *gag/pol* gene is encoded by nucleotides 621 through 5834. Within this sequence, the protein integrase is encoded by nucleotides 4610 through nucleotide 5834. A portion of the *gag/pol* sequence which encodes integrase also encodes env (which begins at nucleotide 5776). Thus, within one embodiment of the invention, the 3' terminal end of the *gag/pol* gene is deleted or truncated in order to prevent crossover with the *env* gene, without effecting the biological activity of the integrase. Within other preferred embodiments, the *gag/pol* gene is deleted at any nucleotide downstream (3') from the beginning of the integrase coding sequence, and preferably prior to the start of the *env* gene sequence. Within one embodiment, the sequence encoding gag/pol is a MoMLV sequence, and the *gag/pol* gene is deleted at any nucleotide between nucleotides 4610 and 5576 (of SEQ. I.D. No. 1), including for example, at nucleotides 5775, 5770, 5765, 5760, 5755, 5750.

Within other embodiments of the invention, the *gag/pol* expression cassette contains sequences encoding gag/pol (and including integrase), while lacking any sequence found in an *env* gene. The phrase "lacking any sequence found in an *env* gene" should be understood to mean that the *gag/pol* expression cassette does not contain at least 20, preferably at least 15, more preferably at least 10, and most preferably less than 8 consecutive nucleotides which are identical to an *env* sequence, and preferably which are found in an *env* expression cassette which will be utilized along with the *gag/pol* expression cassette to form a packaging cell. Such expression cassettes may be readily prepared by selecting codons which are degenerate for integrase, and which do not encode biologically active env. (*See Morgenstern and Land, Nuc. Acids Res. 18*:3587-3596, 1990.)

Within other embodiments of the invention, the *gag/pol* expression cassette contains a heterologous promoter, and/or heterologous polyadenylation sequence. As utilized herein, "heterologous" promoters or polyadenylation sequences refers to promoters or polyadenylation sequences which are from a different source from which the *gag/pol* gene (and preferably the *env* gene and retroviral vector construct) is derived from. Representative examples of suitable promoters include the Cytomegalovirus Immediate Early ("CMV IE") promoter, the Herpes Simplex Virus Thymidine Kinase ("HSVTK") promoter, the Rous Sarcoma Virus ("RSV") promoter, the Adenovirus major-late promoter and the SV 40 promoter. Representative examples

of suitable polyadenylation signals include the SV 40 late polyadenylation signal and the SV40 early polyadenylation signal.

Within preferred aspects of the present invention, *gag/pol* expression cassettes such as those described above will not co-encapsidate along with a replication competent virus. One representative method for determination of co-encapsidation is set forth below in Example 8.

3. *Construction of env expression cassettes*

Within other aspects of the present invention, *env* expression cassettes are provided which, in combination with the *gag/pol* expression cassettes and retroviral vector constructs described above, preclude formation of replication competent virus by homologous recombination, as well as to confer a particular specificity of the resultant vector particle (*e.g.*, amphotropic, ecotropic, xenotropic or polytropic; *see* Figure 17, as well as the discussion above). Briefly, in a wild-type retrovirus the *env* gene encodes two principal proteins, the surface glycoprotein "SU" and the transmembrane protein "TM", which are translated as a polyprotein, and subsequently separated by proteolytic cleavage. Representative examples of the SU and TM proteins are the gp120 protein and gp41 protein in HIV, and the gp70 protein and p15e protein in MoMLV. In some retroviruses, a third protein designated the "R" peptide" of undetermined function, is also expressed from the *env* gene and separated from the polyprotein by proteolytic cleavage. In the Murine Leukemia Virus MoMLV, the R peptide is designated "p2".

A wide variety of *env* expression cassettes may be constructed given the disclosure provided herein, and utilized within the present invention to preclude homologous recombination. Within one aspect of the present invention, *env* expression cassettes are provided comprising a promoter operably linked to an *env* gene, wherein no more than 6, 8, 10, 15, or 20 consecutive retroviral nucleotides are included upstream (5') of and/or contiguous with said *env* gene. Within other aspects of the invention, *env* expression cassettes are provided comprising a promoter operably linked to an *env* gene, wherein the *env* expression cassette does not contain a consecutive sequence of greater than 20, preferably less than 15, more preferably less than 10, and most preferably less than 8 or 6 consecutive nucleotides which are found in a *gag/pol* gene, and in particular, in a *gag/pol* expression cassette that will be utilized along with the *env* expression cassette to create a packaging cell line.

Within another aspect of the present invention, *env* expression cassettes are provided comprising a promoter operably linked to an *env* gene, and a polyadenylation sequence, wherein a 3' terminal end of the *env* gene has been deleted

without effecting the biological activity of env. As utilized herein, the phrase "biological activity of env" refers to the ability of envelop protein to be expressed on the surface of a virus or vector particle, and to allow for a successful infection of a host cell. One practical method for assessing biological activity is to transiently transfect the *env* expression cassette into a cell containing a previously determined functional *gag/pol* expression cassette, and a retroviral vector construct which expresses a selectable marker. A biologically functional *env* expression cassette will allow vector particles produced in that transfected cell, to transmit the selectable marker to a naive sensitive cell such that it becomes resistant to the marker drug selection. Within a preferred embodiment of the invention, the 3' terminal end of the *env* gene is deleted or truncated such that a complete R peptide is not produced by the expression cassette. In the representative example of MoMLV, sequence encoding the R peptide (which begins at nucleotide 7734) is deleted, truncated, or, for example, terminated by insertion of a stop codon at nucleotide 7740, 7745, 7747, 7750, 7755, 7760, 7765, 7770, 7775, 7780, or any nucleotide in between.

Within another aspect of the present invention, *env* expression cassettes are provided which contain a heterologous promoter, and/or heterologous polyadenylation sequence. As utilized herein, "heterologous" promoters or polyadenylation sequences refers to promoters or polyadenylation sequences which are from a different source from which the *gag/pol* gene (and preferably the *env* gene and retroviral vector construct) is derived from. Representative examples of suitable promoters include the CMV IE promoter, the HSVTK promoter, the RSV promoter, the Adenovirus major-late promoter and the SV 40 promoters. Representative examples of suitable polyadenylation signals include the SV 40 late polyadenylation signal and the SV40 early polyadenylation signal.

HETEROLOGOUS SEQUENCES

As noted above, the retroviral vector constructs, *gag/pol* expression cassettes, and *env* expression cassettes of the present invention may contain (and express) one or more heterologous sequences. A wide variety of heterologous sequences may be utilized within the context of the present invention, including for example, cytotoxic genes, antisense sequences, sequences which encode gene products that activate a compound with little or no cytotoxicity (*i.e.*, a "prodrug") into a toxic product, sequences which encode immunogenic portions of disease-associated antigens and sequences which encode immune accessory molecules. Representative examples of cytotoxic genes include the genes which encode proteins such as ricin (Lamb et al., *Eur.*

J. Biochem. 148:265-270, 1985), abrin (Wood et al., *Eur. J. Biochem. 198*:723-732, 1991; Evensen, et al., *J. of Biol. Chem. 266*:6848-6852, 1991: Collins et al., *J. of Biol. Chem. 265*:8665-8669, 1990; Chen et al., *Fed. of Eur. Biochem Soc. 309*:115-118, 1992), diphtheria toxin (Tweten et al., *J. Biol. Chem. 260*:10392-10394, 1985), cholera toxin (Mekalanos et al., *Nature 306*:551-557, 1983; Sanchez & Holmgren, *PNAS 86*:481-485, 1989), gelonin (Stirpe et al., *J. Biol. Chem. 255*:6947-6953, 1980), pokeweed (Irvin, *Pharmac. Ther. 21*:371-387, 1983), antiviral protein (Barbieri et al., *Biochem. J. 203*:55-59, 1982; Irvin et al., *Arch. Biochem. & Biophys. 200*:418-425, 1980; Irvin, *Arch. Biochem. & Biophys. 169*:522-528, 1975), tritin, Shigella toxin (Calderwood et al., *PNAS 84*:4364-4368, 1987; Jackson et al., *Microb. Path. 2*:147-153, 1987), and Pseudomonas exotoxin A (Carroll and Collier, *J. Biol. Chem. 262*:8707-8711, 1987).

Within further embodiments of the invention, antisense RNA may be utilized as a cytotoxic gene in order to induce a potent Class I restricted response. Briefly, in addition to binding RNA and thereby preventing translation of a specific mRNA, high levels of specific antisense sequences may be utilized to induce the increased expression of interferons (including gamma-interferon), due to the formation of large quantities of double-stranded RNA. The increased expression of gamma interferon, in turn, boosts the expression of MHC Class I antigens. Preferred antisense sequences for use in this regard include actin RNA, myosin RNA, and histone RNA. Antisense RNA which forms a mismatch with actin RNA is particularly preferred.

Within other embodiments of the invention, antisense sequences are provided which inhibit, for example, tumor cell growth, viral replication, or a genetic disease by preventing the cellular synthesis of critical proteins needed for cell growth. Examples of such antisense sequences include antisense thymidine kinase, antisense dihydrofolate reductase (Maher and Dolnick, *Arch. Biochem. & Biophys. 253*:214-220, 1987; Bzik et al., *PNAS 84*:8360-8364, 1987), antisense HER2 (Coussens et al., *Science 230*:1132-1139, 1985), antisense ABL (Fainstein, et al., *Oncogene 4*:1477-1481, 1989), antisense Myc (Stanton et al., *Nature 310*:423-425, 1984) and antisense *ras*, as well as antisense sequences which block any of the enzymes in the nucleotide biosynthetic pathway.

Within other aspects of the invention, retroviral vector constructs, *gag/pol* expression cassettes and *env* expression cassettes are provided which direct the expression of a gene product that activates a compound with little or no cytotoxicity (*i.e.*, a "prodrug") into a toxic product. Representative examples of such gene products include varicella zoster virus thymidine kinase (VZVTK), herpes simplex virus thymidine

kinase (HSVTK) (Field et al., *J. Gen. Virol. 49*:115-124, 1980; Munir et al., *Protein Engineering* 7(1):83-89, 1994; Black and Loeb, *Biochem 32*(43):11618-11626, 1993), and *E. coli.* guanine phosphoribosyl transferase (*see* U.S. Patent Application Serial No. 08/155,944, entitled "Compositions and Methods for Utilizing Conditionally Lethal Genes," filed November 18, 1993; *see also* WO 93/10218 entitled "Vectors Including Foreign Genes and Negative Selection Markers", WO 93/01281 entitled "Cytosine Deaminase Negative Selection System for Gene Transfer Techniques and Therapies", WO 93/08843 entitled "Trapped Cells and Use Thereof as a Drug", WO 93/08844 entitled "Transformant Cells for the Prophylaxis or Treatment of Diseases Caused by Viruses, Particularly Pathogenic Retroviruses", and WO 90/07936 entitled "Recombinant Therapies for Infection and Hyperproliferative Disorders.") Within preferred embodiments of the invention, the retroviral vector constructs direct the expression of a gene product that activates a compound with little or no cytotoxicity into a toxic product in the presence of a pathogenic agent, thereby affecting localized therapy to the pathogenic agent (*see* WO 94/13304).

Within one embodiment of the invention, retroviral vector constructs are provided which direct the expression of a HSVTK gene downstream, and under the transcriptional control of an HIV promoter (which is known to be transcriptionally silent except when activated by HIV tat protein). Briefly, expression of the tat gene product in human cells infected with HIV and carrying the vector construct causes increased production of HSVTK. The cells (either *in vitro* or *in vivo*) are then exposed to a drug such as ganciclovir, acyclovir or its analogues (FIAU, FIAC, DHPG). Such drugs are known to be phosphorylated by HSVTK (but not by cellular thymidine kinase) to their corresponding active nucleotide triphosphate forms. Acyclovir and FIAU triphosphates inhibit cellular polymerases in general, leading to the specific destruction of cells expressing HSVTK in transgenic mice (*see* Borrelli et al., *Proc. Natl. Acad. Sci. USA 85*:7572, 1988). Those cells containing the recombinant vector and expressing HIV tat protein are selectively killed by the presence of a specific dose of these drugs.

Within further aspects of the present invention, retroviral vector constructs, *gag/pol* expression cassettes and *env* expression cassettes of the present invention may also direct the expression of one or more sequences which encode immunogenic portions of disease-associated antigens. As utilized within the context of the present invention, antigens are deemed to be "disease-associated" if they are either associated with rendering a cell (or organism) diseased, or are associated with the disease-state in general but are not required or essential for rendering the cell diseased. In addition, antigens are considered to be "immunogenic" if they are capable, under

appropriate conditions, of causing an immune response (either cell-mediated or humoral). Immunogenic "portions" may be of variable size, but are preferably at least 9 amino acids long, and may include the entire antigen.

A wide variety of "disease-associated" antigens are contemplated within the scope of the present invention, including for example immunogenic, non-tumorigenic forms of altered cellular components which are normally associated with tumor cells (*see* WO 93/10814). Representative examples of altered cellular components which are normally associated with tumor cells include ras* (wherein "*" is understood to refer to antigens which have been altered to be non-tumorigenic), p53*, Rb*, altered protein encoded by Wilms' tumor gene, ubiquitin*, mucin, protein encoded by the DCC. APC, and MCC genes, as well as receptors or receptor-like structures such as neu, thyroid hormone receptor, Platelet Derived Growth Factor ("PDGF") receptor, insulin receptor, Epidermal Growth Factor ("EGF") receptor, and the Colony Stimulating Factor ("CSF") receptor.

"Disease-associated" antigens should also be understood to include all or portions of various eukaryotic, prokaryotic or viral pathogens. Representative examples of viral pathogens include the Hepatitis B Virus ("HBV") and Hepatitis C Virus ("HCV"; *see* WO 93/15207), Human Papiloma Virus ("HPV"; *see* WO 92/05248; WO 90/10459; EPO 133,123), Epstein-Barr Virus ("EBV"; *see* EPO 173,254; JP 1,128,788; and U.S. Patent Nos. 4,939,088 and 5,173,414), Feline Leukemia Virus ("FeLV"; *see* WO 93/09070; EPO 377,842; WO 90/08832; WO 93/09238), Feline Immunodeficiency Virus ("FIV"; U.S. Patent No. 5,037,753; WO 92/15684; WO 90/13573; and JP 4,126,085), HTLV I and II, and Human Immunodeficiency Virus ("HIV"; *see* WO 93/02805).

Within other aspects of the present invention, the retroviral vector constructs, *gag/pol* expression cassettes and *env* expression cassettes described above may also direct the expression of one or more immune accessory molecules. As utilized herein, the phrase "immune accessory molecules" refers to molecules which can either increase or decrease the recognition, presentation or activation of an immune response (either cell-mediated or humoral). Representative examples of immune accessory molecules include α interferon, β interferon, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7 (U.S. Patent No. 4,965,195), IL-8, IL-9, IL-10, IL-11, IL-12 (Wolf et al., *J. Immun. 46*:3074, 1991; Gubler et al., *PNAS 88*:4143, 1991; WO 90/05147; EPO 433,827), IL-13 (WO 94/04680), IL-14, IL-15, GM-CSF, M-CSF-1, G-CSF, CD3 (Krissanen et al., *Immunogenetics 26*:258-266, 1987), CD8, ICAM-1 (Simmons et al., *Nature 331*:624-627, 1988), ICAM-2 (Singer, *Science 255*: 1671, 1992), β-microglobulin (Parnes et al.,

PNAS 78:2253-2257, 1981), LFA-1 (Altmann et al., *Nature 338*: 521, 1989), LFA3 (Wallner et al., *J. Exp. Med. 166*(4):923-932, 1987), HLA Class I, HLA Class II molecules, B7 (Freeman et al., *J. Immun. 143*:2714, 1989), and B7-2. Within a preferred embodiment, the heterologous gene encodes gamma-interferon.

Within preferred aspects of the present invention, the retroviral vector constructs described herein may direct the expression of more than one heterologous sequence. Such multiple sequences may be controlled either by a single promoter, or preferably, by additional secondary promoters (*e.g.*, Internal Ribosome Binding Sites or "IRBS"). Within preferred embodiments of the invention, retroviral vector constructs direct the expression of heterologous sequences which act synergistically. For example, within one embodiment retroviral vector constructs are provided which direct the expression of a molecule such as IL-15, IL-12, IL-2, gamma interferon, or other molecule which acts to increase cell-mediated presentation in the T_H1 pathway, along with an immunogenic portion of a disease-associated antigen. In such embodiments, immune presentation and processing of the disease-associated antigen will be increased due to the presence of the immune accessory molecule.

Within other aspects of the invention, retroviral vector constructs are provided which direct the expression of one or more heterologous sequences which encode "replacement" genes. As utilized herein, it should be understood that the term "replacement genes" refers to a nucleic acid molecule which encodes a therapeutic protein that is capable of preventing, inhibiting, stabilizing or reversing an inherited or noninherited genetic defect. Representative examples of such genetic defects include disorders in metabolism, immune regulation, hormonal regulation, and enzymatic or membrane associated structural function. Representative examples of diseases caused by such defects include Cystic Fibrosis ("CF"; *see* Dorin et al., *Nature 326*:614,), Parkinson's Disease, Adenosine Deaminase deficiency ("ADA"; Hahma et al., *J. Bact. 173*:3663-3672, 1991), β-globin disorders, Hemophilia A & B (Factor VIII-deficiencies; *see* Wood et al., *Nature 312*:330, 1984), Gaucher disease, diabetes, forms of gouty arthritis and Lesch-Nylan disease (due to "HPRT" deficiencies; *see* Jolly et al., *PNAS 80*:477-481, 1983) and Familial Hypercholesterolemia (LDL Receptor mutations; *see* Yamamoto et al., *Cell 39*:27-38, 1984).

Sequences which encode the above-described heterologous genes may be readily obtained from a variety of sources. For example, plasmids which contain sequences that encode immune accessory molecules may be obtained from a depository such as the American Type Culture Collection (ATCC, Rockville, Maryland), or from commercial sources such as British Bio-Technology Limited (Cowley, Oxford England).

Representative sources sequences which encode the above-noted immune accessory molecules include BBG 12 (containing the GM-CSF gene coding for the mature protein of 127 amino acids), BBG 6 (which contains sequences encoding gamma interferon), ATCC No. 39656 (which contains sequences encoding TNF), ATCC No. 20663 (which contains sequences encoding alpha interferon), ATCC Nos. 31902, 31902 and 39517 (which contains sequences encoding beta interferon), ATCC No 67024 (which contains a sequence which encodes Interleukin-1), ATCC Nos. 39405, 39452, 39516, 39626 and 39673 (which contains sequences encoding Interleukin-2), ATCC Nos. 59399, 59398, and 67326 (which contain sequences encoding Interleukin-3), ATCC No. 57592 (which contains sequences encoding Interleukin-4), ATCC Nos. 59394 and 59395 (which contain sequences encoding Interleukin-5), and ATCC No. 67153 (which contains sequences encoding Interleukin-6). It will be evident to one of skill in the art that one may utilize either the entire sequence of the protein, or an appropriate portion thereof which encodes the biologically active portion of the protein.

Alternatively, known cDNA sequences which encode cytotoxic genes or other heterologous genes may be obtained from cells which express or contain such sequences. Briefly, within one embodiment mRNA from a cell which expresses the gene of interest is reverse transcribed with reverse transcriptase using oligo dT or random primers. The single stranded cDNA may then be amplified by PCR (*see* U.S. Patent Nos. 4,683,202, 4,683,195 and 4,800,159. *See also* PCR Technology: Principles and Applications for DNA Amplification, Erlich (ed.), Stockton Press, 1989 all of which are incorporated by reference herein in their entirety) utilizing oligonucleotide primers complementary to sequences on either side of desired sequences. In particular, a double stranded DNA is denatured by heating in the presence of heat stable Taq polymerase, sequence specific DNA primers, ATP, CTP, GTP and TTP. Double-stranded DNA is produced when synthesis is complete. This cycle may be repeated many times, resulting in a factorial amplification of the desired DNA.

Sequences which encode the above-described genes may also be synthesized, for example, on an Applied Biosystems Inc. DNA synthesizer (*e.g.*, ABI DNA synthesizer model 392 (Foster City, California)).

PREPARATION OF RETROVIRAL PACKAGING CELL LINES, AND GENERATION OF RECOMBINANT VIRAL PARTICLES

As noted above, the *gag/pol* expression cassettes and *env* expression cassettes of the present invention may be used to generate transduction competent retroviral vector particles by introducing them into an appropriate parent cell line in

order to create a packaging cell line, followed by introduction of a retroviral vector construct, in order to create a producer cell line (*see* WO 92/05266). Such packaging cell lines, upon introduction of an N2-type vector construct (Armentano et al., *J. of Vir. 61*(5):1647-1650, 1987) produce a titer of greater than 10^5 cfu/ml, and preferably greater than 10-fold, 20-fold, 50-fold, or 100-fold higher titer than similar transduced PA317 cells (Miller and Buttimore, *Mol. and Cell. Biol. 6*(8):2895-2902, 1986).

Within one aspect of the present invention, methods for creating packaging cell lines are provided, comprising the steps of (a) introducing a *gag/pol* expression cassette according into an animal cell, (b) selecting a cell containing a *gag/pol* expression cassette which expresses high levels of *gag/pol*, (c) introducing an *env* expression cassette into the selected cell, and (d) selecting a cell which expresses high levels of *env*, and thereby creating the packaging cell. Within other aspects of the present invention, methods for creating packaging cell lines are provided comprising the steps of (a) introducing an *env* expression cassette into an animal cell (b) selecting a cell which expresses high levels of *env*, (c) introducing a *gag/pol* expression cassette into the selected cell, and (d) selecting a cell containing a *gag/pol* expression cassette which expresses high levels of *gag/pol*, and thereby creating the packaging cell. As utilized herein, it should be understood that "high" levels of gag/pol or env refers to packaging cells which produce at least z times greater gag/pol or env protein than PA317 cells, wherein z is selected from the group consisting of 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10.

A wide variety of animal cells may be utilized to prepare the packaging cells of the present invention, including for example human, macaque, dog, rat and mouse cells. Particularly preferred cell lines for use in the preparation of packaging cell lines of the present invention are those that lack genomic sequences which are homologous to the retroviral vector construct, *gag/pol* expression cassette and *env* expression cassette to be utilized. Methods for determining homology may be readily accomplished by, for example, hybridization analysis (*see Martin et al., PNAS 78*:4892-4896, 1981; *see also* WO 92/05266).

Expression cassettes of the present invention may be introduced into cells by numerous techniques, including for example, transfection by various physical methods, such as electroporation, DEAE dextran, lipofection (Felgner et al., *Proc. Natl. Acad. Sci. USA 84*:7413-7417, 1989), direct DNA injection (Acsadi et al., *Nature 352*:815-818, 1991); microprojectile bombardment (Williams et al., *PNAS 88*:2726-2730, 1991), liposomes of several types (*see e.g.*, Wang et al., *PNAS 84*:7851-7855, 1987); $CaPO_4$ (Dubensky et al., *PNAS 81*:7529-7533, 1984), DNA ligand (Wu et al, *J. of Biol. Chem. 264*:16985-16987, 1989), administration of nucleic acids alone (WO

90/11092), or administration of DNA linked to killed adenovirus (Curiel et al., *Hum. Gene Ther. 3*: 147-154, 1992).

Producer cell lines (also called vector-producing lines or "VCLs") may then be readily prepared by introducing a retroviral vector construct as described above, into a packaging cell line. Within preferred embodiments of the invention, producer cell lines are provided comprising a *gag/pol* expression cassette, an *env* expression cassette, and a retroviral vector construct, wherein the *gag/pol* expression cassette, *env* expression cassette and retroviral vector construct lack a consecutive sequence of greater than 20, preferably 15, more preferably 10, and most preferably 10 or 8 nucleotides in common.

PHARMACEUTICAL COMPOSITIONS

Within another aspect of the invention, pharmaceutical compositions are provided, comprising a recombinant viral particle as described above, in combination with a pharmaceutically acceptable carrier or diluent. Such pharmaceutical compositions may be prepared either as a liquid solution, or as a solid form (*e.g.*, lyophilized) which is suspended in a solution prior to administration. In addition, the composition may be prepared with suitable carriers or diluents for topical administration, injection, or oral, nasal, vaginal, sub-lingual, inhalant or rectal administration.

Pharmaceutically acceptable carriers or diluents are nontoxic to recipients at the dosages and concentrations employed. Representative examples of carriers or diluents for injectable solutions include water, isotonic saline solutions which are preferably buffered at a physiological pH (such as phosphate-buffered saline or Tris-buffered saline), mannitol, dextrose, glycerol, and ethanol, as well as polypeptides or proteins such as human serum albumin. A particularly preferred composition comprises a retroviral vector construct or recombinant viral particle in 10 mg/ml mannitol, 1 mg/ml HSA, 20 mM Tris, pH 7.2, and 150 mM NaCl. In this case, since the recombinant vector represents approximately 1 mg of material, it may be less than 1% of high molecular weight material, and less than 1/100,000 of the total material (including water). This composition is stable at -70°C for at least six months.

Pharmaceutical compositions of the present invention may also additionally include factors which stimulate cell division, and hence, uptake and incorporation of a recombinant retroviral vector. Representative examples include Melanocyte Stimulating Hormone (MSH), for melanomas or epidermal growth factor for breast or other epithelial carcinomas.

Particularly preferred methods and compositions for preserving recombinant viruses are described in U.S. applications entitled "Methods for Preserving Recombinant Viruses" (*see* WO 94/11414).

METHODS OF ADMINISTRATION

Within other aspects of the present invention, methods are provided for inhibiting or destroying pathogenic agents in a warm-blooded animal, comprising administering to a warm-blooded animal a recombinant viral particle as described above, such that the pathogenic agent is inhibited or destroyed. Within various embodiments of the invention, recombinant viral particles may be administered *in vivo*, or *ex vivo*. Representative routes for *in vivo* administration include intradermally ("i.d."), intracranially ("i.c."), intraperitoneally ("i.p."), intrathecally ("i.t."), intravenously ("i.v."), subcutaneously ("s.c."), intramuscularly ("i.m.") or even directly into a tumor.

Alternatively, the cytotoxic genes, antisense sequences, gene products, retroviral vector constructs or viral particles of the present invention may also be administered to a warm-blooded animal by a variety of other methods. Representative examples include transfection by various physical methods, such as lipofection (Felgner et al., *Proc. Natl. Acad. Sci. USA 84*:7413-7417, 1989), direct DNA injection (Acsadi et al., *Nature 352*:815-818, 1991); microprojectile bombardment (Williams et al., *PNAS 88*:2726-2730, 1991); liposomes of several types (*see e.g.*, Wang et al., *PNAS 84*:7851-7855, 1987); $CaPO_4$ (Dubensky et al., *PNAS 81*:7529-7533, 1984); DNA ligand (Wu et al, *J. of Biol. Chem. 264*:16985-16987, 1989); administration of nucleic acids alone (WO 90/11092); or administration of DNA linked to killed adenovirus (Curiel et al., *Hum. Gene Ther. 3*: 147-154, 1992).

Within a preferred aspect of the present invention, retroviral particles (or retroviral vector constructs alone) may be utilized in order to directly treat pathogenic agents such as a tumor. Within preferred embodiments, the retroviral particles or retroviral vector constructs described above may be directly administered to a tumor, for example, by direct injection into several different locations within the body of tumor. Alternatively, arteries which serve a tumor may be identified, and the vector injected into such an artery, in order to deliver the vector directly into the tumor. Within another embodiment, a tumor which has a necrotic center may be aspirated, and the vector injected directly into the now empty center of the tumor. Within yet another embodiment, the retroviral vector construct may be directly administered to the surface of the tumor, for example, by application of a topical pharmaceutical composition

containing the retroviral vector construct, or preferably, a recombinant retroviral particle.

Within another aspect of the present invention, methods are provided for inhibiting the growth of a selected tumor in a warm-blooded animal, comprising the steps of (a) removing tumor cells associated with the selected tumor from a warm-blooded animal, (b) infecting the removed cells with a retroviral vector construct which directs the expression of at least one anti-tumor agent, and (c) delivering the infected cells to a warm-blooded animal, such that the growth of the selected tumor is inhibited by immune responses generated against the gene-modified tumor cell. Within the context of the present invention, "inhibiting the growth of a selected tumor" refers to either (1) the direct inhibition of tumor cell division, or (2) immune cell mediated tumor cell lysis, or both, which leads to a suppression in the net expansion of tumor cells. Inhibition of tumor growth by either of these two mechanisms may be readily determined by one of ordinary skill in the art based upon a number of well known methods (*see* U.S. Serial No. 08/032,846). Examples of compounds or molecules which act as anti-tumor agents include immune accessory molecules, cytotoxic genes, and antisense sequences as discussed above (*see also* U.S. Serial No. 08/032,846).

Cells may be removed from a variety of locations including, for example, from a selected tumor. In addition, within other embodiments of the invention, a vector construct may be inserted into non-tumorigenic cells, including for example, cells from the skin (dermal fibroblasts), or from the blood (*e.g.*, peripheral blood leukocytes). If desired, particular fractions of cells such as a T cell subset or stem cells may also be specifically removed from the blood (*see,* for example, PCT WO 91/16116, an application entitled "Immunoselection Device and Method"). Vector constructs may then be contacted with the removed cells utilizing any of the above-described techniques, followed by the return of the cells to the warm-blooded animal, preferably to or within the vicinity of a tumor. Within one embodiment of the present invention, subsequent to removing tumor cells from a warm-blooded animal, a single cell suspension may be generated by, for example, physical disruption or proteolytic digestion. In addition, division of the cells may be increased by addition of various factors such as melanocyte stimulating factor for melanomas or epidermal growth factor for breast carcinomas, in order to enhance uptake, genomic integration and expression of the recombinant viral vector.

Within the context of the present invention, it should be understood that the removed cells may not only be returned to the same animal, but may also be utilized to inhibit the growth of selected tumor cells in another, allogeneic, animal. In such a

case it is generally preferable to have histocompatibility matched animals (although not always, *see, e.g.*, Yamamoto et al., "Efficacy of Experimental FIV Vaccines," 1st International Conference of FIV Researchers, University of California at Davis, September 1991).

The above-described methods may additionally comprise the steps of depleting fibroblasts or other non-contaminating tumor cells subsequent to removing tumor cells from a warm-blooded animal, and/or the step of inactivating the cells, for example, by irradiation.

As noted above, within certain aspects of the present invention, several anti-tumor agents may be administered either concurrently or sequentially, in order to inhibit the growth of a selected tumor in accordance with the methods of the present invention. For example, within one embodiment of the invention, an anti-tumor agent such as γ-IFN may be co-administered or sequentially administered to a warm-blooded animal along with other anti-tumor agents such as IL-2, or IL-12, in order to inhibit or destroy a pathogenic agent. Such therapeutic compositions may be administered directly utilizing a single vector construct which directs the expression of at least two anti-tumor agents, or, within other embodiments, expressed from independent vector constructs. Alternatively, one anti-tumor agent (*e.g.*, γ-IFN) may be administered utilizing a vector construct, while other tumor agents (*e.g.*, IL-2) are administered directly (*e.g.*, as a pharmaceutical composition intravenously).

Within a particularly preferred embodiment, retroviral vector constructs which deliver and express both a γ-IFN gene and another gene encoding IL-2, may be administered to the patient. In such constructs, one gene may be expressed from the retrovector LTR and the other may utilize an additional transcriptional promoter found between the LTRs, or may be expressed as a polycistronic mRNA, possibly utilizing an internal ribosome binding site. After *in vivo* gene transfer, the patient's immune system is activated due to the expression of γ-IFN. Infiltration of the dying tumor with inflammatory cells, in turn, increases immune presentation and further improves the patient's immune response against the tumor.

Within other aspects of the present invention, methods are provided for generating an immune response against an immunogenic portion of an antigen, in order to prevent or treat a disease (*see, e.g.*, U.S. Serial Nos. 08/104,424; 08/102,132, 07/948,358; 07/965,084), for suppressing graft rejection, (*see* U.S. Serial No. 08/116,827), for suppressing an immune response (*see* U.S. Serial No. 08/116,828), and for suppressing an autoimmune response (*see* U.S. Serial No. 08/116,983).

As will be understood by one of ordinary skill in the art given the disclosure provided herein, any of the retroviral vector constructs described herein may be delivered not only as a recombinant viral particle, but as direct nucleic acid vectors. Such vectors may be delivered utilizing any appropriate physical method of gene transfer, including for example, those which have been discussed above.

The following examples are offered by way of illustration, and not by way of limitation.

EXAMPLE 1

CONSTRUCTION OF RETROVECTOR BACKBONES

A. Preparation of a Retroviral vector construct That Does Not Contain an Extended Packaging Sequence (Ψ)

This example describes the construction of a retroviral vector construct using site-specific mutagenesis. Within this example, a MoMLV retroviral vector construct is prepared wherein the packaging signal "Ψ" of the retrovector is terminated at basepair 617 of SEQ ID NO: 1, thereby eliminating the ATG start of *gag*. Thus, no crossover can occur between the retroviral vector construct and the *gag/pol* expression cassette which is described below in Example 3.

Briefly, pMLV-K (Miller, *J. Virol* 49:214-222, 1984 - an infectious clone derived from pMLV-1 Shinnick et al., *Nature, 293*:543-548, 1981) is digested with *Eco*57I, and a 1.9kb fragment is isolated. (*Eco*57I cuts upstream from the 3' LTR, thereby removing all *env* coding segments from the retroviral vector construct.) The fragment is then blunt ended with T4 polymerase (New England Biolabs), and all four deoxynucleotides, and cloned into the *Eco*RV site of phagemid pBluescript II KS+ (Stratagene, San Diego, Calif.). This procedure yields two constructs, designated pKS2+Eco57I-LTR(+) (Figure 1) and pKS2+Eco57I-LTR(-) (Figure 2), which are screened by restriction analysis. When the (+) single stranded phagemid is generated, the sense sequence of MoMLV is isolated.

A new *Eco*RI site is then created in construct pKS2+*Eco*57I-LTR(+) in order to remove the ATG start codon of *gag*. In particular, an *Eco*RI site is created using the single stranded mutagenesis method of Kunkle (*PNAS 82*:488, 1985). pKS2+*Eco*57I-LTR(+) is a pBluescript™ II + phagemid (Strategene, San Diego, Calif.) containing an *Eco*57I fragment from pMLV-K. It includes the MoMLV LTR and downstream sequence to basepair 1378. When single stranded phagemid is generated the sense sequence of MoMLV is isolated. The oligonucleotide, 5'-GGT AAC AGT CTG GCC CGA ATT CTC AGA CAA ATA CAG (SEQ ID NO: 2), is created and used to generate an *Eco*RI site at basepairs 617-622. This construct is designated pKS2+LTR-EcoRI (Figure 3).

B. Substitution of Nonsense Codons in the Extended Packaging Sequence (Ψ+)

This example describes modification of the extended packaging signal (Ψ+) by site-specific mutagenesis. In particular, the modification will substitute a stop

codon, TAA, at the normal ATG start site of *gag* (position 631-633 of SEQ ID NO: 1), and an additional stop codon TAG at position 637-639 of SEQ ID NO: 1.

Briefly, an *Eco*571 - *Eco*RI fragment (MoMLV basepairs 7770 to approx. 1040) from pN2 (Amentano et al., J. Virol. 61:1647-1650, 1987) is first cloned into pBluescript II KS+ phagemid at the *Sac*II and *Eco*RI sites (compatible). Single stranded phagemid containing antisense MoMLV sequence, is generated using helper phage M13K07 (Stratagene, San Diego, Calif.). The oligonucleotide 5'-CTG TAT TTG TCT GAG AAT TAA GGC TAG ACT GTT ACC AC (SEQ ID NO: 3) is synthesized, and utilize according to the method of Kunkle as described above, in order to modify the sequence within the Ψ region to encode stop codons at nucleotides 631-633 and 637-639.

C. Removal of Retroviral Packaging Sequence Downstream from the 3' LTR

Retroviral packaging sequence which is downstream from the 3' LTR is deleted essentially as described below. Briefly, pKS2+Eco571-LTR(-) (Figure 2) is digested with *Ball* and *Hin*cII, and relegated excluding the *Ball* to *Hin*cII DNA which contains the packaging region of MoMLV.

D. Construction of Vector Backbones

Constructs prepared in sections A and C above, or alternatively from sections B and C above, are combined with a plasmid vector as described below, in order to create a retrovector backbone containing all elements required *in cis*, and excluding all sequences of 8 nucleic acids or more contained in the retroviral portion of the *gag-pol* and *env* expression elements (*see* Examples 3 and 4).

1. Parts A and C are combined as follows: The product of A is digested with *Nhe*I and *Eco*RI, and a 1034 basepair fragment containing the LTR and minimal Ψ is isolated. The fragment is ligated into the product of part C at the unique (compatible) restriction sites *Spe*I and *Eco*RI. The resultant construct is designated pR1 (Figure 4)

2. Parts B and C are combined as follows: The product of B is digested with *Nhe*I and *Eco*RI and a 1456 basepair fragment containing the LTR and modified Ψ+ region is isolated. The fragment is ligated into the product of C at the unique (compatible) restriction sites *Spe*I and *Eco*RI. The resultant construct is designated pR2 (Figure 5).

EXAMPLE 2

INSERTION OF A GENE OF INTEREST INTO PR1 AND PR2

This example describes the insertion of a gene of interest, gp120, gp41, and rev along with a selectable marker into either pR1 or pR2. Briefly, the sequence encoding gp120, gp41 and rev is taken from construct pKT1 (Figure 6; *see also* Chada et al., *J. Vir. 67*:3409-3417, 1993); note that this vector is also referred to as N2IIIBenv. In particular, pKT1 is first digested at the unique *Asn*II site (position 5959). The ends are blunted, and an *Xho* 1 linker is ligated at that site. (New England Biolabs). The construct is then digested with *Xho* 1, and a 4314 bp fragment containing HIV envelope (gp120 and gp41), rev, SV40 early promoter and G418 resistance genes is isolated.

pR1 or pR2 is digested at the unique *Eco* R1 restriction site, blunted, and *Sal* 1 linkers (New England Biolabs) are ligated in. The 4314 bp KT1 fragment is then ligated into pR1 or pR2 at the new *Sal* I sites, and the correct orientation is determined (*see* Figures 7 and 8). In both of these constructs, (pR1-HIVenv and pR2-HIVenv) the HIV genes are expressed from the MLV LTR, and G418 resistance is expressed from the SV40 promoter.

EXAMPLE 3

CONSTRUCTION OF *GAG-POL* EXPRESSION CASSETTES

A. Construction of an Expression Cassette Backbone, pHCMU-PA

A vector is first created in order to form the backbone for both the *gag/pol* and *env* expression cassettes. Briefly, pBluescript SK- phagemid (Stratagene, San Diego, Calif.; GenBank accession number 52324; referred to as "SK-") is digested with *Spe*I and blunt ended with Klenow. A blunt end *Dra*I fragment of SV40 (Fiers et al., "Complete nucleotide sequence of SV40 DNA" *Nature 273*:113-120, 1978) from DraI (bp 2366) to DraI (bp2729) is then inserted into SK-, and a construct isolated in which the SV40 late polyadenylation signal is oriented opposite to the LacZ gene of SK-. This construct is designated SK-SV40A.

A Human Cytomegalovirus Major Immediate Early Promoter ("HCMV-IE"; Boshart et al., *Cell 41*:521-530, 1985) (*Hin*cII, bp 140, to *Eag*I, bp814) is isolated after digestion with *Hin*cII and *Eag*I, and the *Eag*I site blunt ended. The 674 blunt ended fragment is ligated into SK-SV40A. The final construct, designated pHCMV-PA is then isolated (*see* Figure 11). This construct contains the HCMV promoter oriented

in opposite orientation to the LacZ gene, and upstream from the late polyadenylation signal of SV40.

B. Creation of New Codons for the 5' *Gag*

This example describes *gag/pol* expression cassettes that lack non-coding sequences upstream from the *gag* start, thereby reducing recombination potential between the *gag-pol* expression element and Ψ+ sequence of a retroviral vector construct, and inhibiting co-packaging of the *gag-pol* expression element along with the retrovector. In order to construct such an expression cassette, 448 bp of DNA is synthesized with the following features: 5' ATATATATATCGAT(*Cla*I site)ACCATG(start codon, position 621) (SEQ ID NO: 4), followed by 410 bp encoding 136+ amino acid residues using alternative codons (*see* Figures 9 and 10), followed by GGCGCC(*Nar*1 site)AAACCTAAAC 3' (SEQ ID NO: 5).

Briefly, each of oligos 15 through 24 (set forth below in Table 1) are added to a PCR reaction tube such that the final concentration for each is 1 μM. Oligos 25 and 26 are added to the tube such that the final concentration for each is 3 μM. 1.2 μL of 2.5 mM stock deoxynucleotide triphosphates (dG, dA, dT, dC) are added to the tube. 5 μL of 10X PCR buffer (Perkin Elmer). Water is added to a final volume of 50 μL. Wax beads are added and melted over the aqueous layer at 55°C and then cooled to 22°C. A top aqueous layer is added as follows: 5 μL 10X PCR buffer, 7.5 μL dimethylsulfoxide, 1.5 μL Taq polymerase (Perkin-Elmer) and 36 μL water. Forty cycles of PCR are then performed as follows: 94°C, 30 seconds; 56°C, 30 seconds; and 72°C, 30 seconds. The PCR product is stored at -20°C until assembly of the *gag/pol* expression cassette.

Table 1

SEQ. ID. No.	Sequence
15	5' ATA TAT ATA TAT CGA TAC CAT GGG GCA AAC CGT GAC TAC CCC TCT GTC CCT CA C ACT GGC CCA A 3'
16	5' TTG ATT ATG GGC AAT TCT TTC CAC GTC CTT CCA ATG GCC CAG TGT GAG GGA C 3'
17	5' AGA ATT GCC CAT AAT CAA AGC GTG GAC GTC AAA AAA CGC AGG TGG GT G ACA TTT TGT AGC GCC GAG TGG CCC 3'

18 5' AAG TTC CAT CCC TAG GCC AGC CAA CAT TGA ATG TGG GCC ACT CGG CGC
TAC A 3'

19 5' GGC CTA GGG ATG GAA CTT TCA ATC GCG ATC TGA TTA CTC AAG TGA AA A TTA AAG TGT TCA GCC CCG GAC CCC 3'

20 5' GTG ACA ATA TAA GGA ACT TGA TCG GGA TGG CCG TGG GGT CCG GGG CTG
AAC A 3'

21 5' AGT TCC TTA TAT TGT CAC ATC GGA GGC TCT CGC TTT CGA TCC ACC ACC TTG GGT GAA ACC ATT CGT GCA TCC 3'

22 5' AGG AGC GCT GGG TGG GAG GGG TGG AGG TGG TTT GGG ATG CAC GAA TGG TTT C 3'

23 5' CTC CCA CCC AGC GCT CCT AGC CTG CCC TTG GAG CCC CCA CGA AGC ACA CCA CCC AGG AGC AGC TTG TAC CCT 3'

24 5' GTT TAG GTT TGG CGC CGA GGC TGG GGG TCA GAG CAG GGT ACA AGC TGC
TCC T 3'

25 5' ATA TAT ATA TAT CGA TAC C 3'

26 5' GTT TAG GTT TGG CGC CGA GG 3'

C. Creation of a New 3' End for *Pol*

In order to prepare a *gag/pol* expression cassette which expresses full length *gag/pol*, pCMV*gag/pol* is constructed. Briefly, MoMLV sequence from *Pst*I (BP567) to *Nhe*I (bp 7847) is cloned into the *Pst*I-*Xba*I sites of pUC19 (New England Biolabs). The resultant intermediate is digested with *Hin*dIII and *Xho*I, and a 1008 bp fragment containing the *gag* leader sequence is isolated. The same intermediate is also digested with *Xho*I and *Sca*I, and a 4312 bp fragment containing the remaining *gag* and *pol* sequences is isolated. The two isolated fragments are then cloned into the *Hin*dIII and *Sma*I sites of pHCMV-PA, described above. The resultant construct, designated CMV *gag/pol* (Figure 12) expresses MoMLV *gag* and *pol* genes.

In order to truncate the 3' end of the *pol* gene found in pCMV *gag-pol*, a 5531 basepair *Sna*BI - *Xma*I fragment containing a portion of the CMV IE promoter and all of *gag-pol* except the final 28 codons, is isolated from pCMV *gag-pol*. This fragment is cloned into the *Sna*BI and *Xma*I sites of pHCMV-PA. This construct expresses five new amino acids at the carboxy-terminus (Ser-Lys-Asn-Tyr-Pro) (SEQ ID NO: 6) (pCMV gpSma).

Alternatively, these five amino acids may be eliminated by digesting pCMVgp *Sma*I with *Sma*I and adding an *Nhe*I (termination codons in three phases) linker (5' - CTA GCT AGC TAG SEQ ID NO: 14; New England Biolabs) at the end of the truncated *pol* sequence. This construct is designated pCMV gp Nhe. Both of these constructs eliminates potential crossover between *gag/pol* and *env* expression cassettes.

D. *Gag-Pol* Expression Cassette

Parts B and C from above are combined to provide an expression vector containing a CMV IE promoter, *gag-pol* sequence starting from the new *Cla*I site (followed by ACC ATG and 412 bp of alternative or "wobble" *gag* coding sequence) and terminating at the *Sma*I site (MoMLV position 5750) followed by an SV40 polyadenylation signal, essentially as described below. Briefly, the approximately 451 bp double stranded wobble fragment from part A is ligated into pCR™II TA cloning vector (Invitrogen Corp.). The wobble PCR product naturally contains a 3' A-overhang at each end, allowing for cloning into the 3' T-overhang of pCR™II. The 422 bp *Cla*I -*Nar*I wobble fragment from the pCR™II clone is removed and is ligated into the *Cla*I (Position 679, Figure pCMV gp Sma) and *Nar*I (Position 1585) sites of pCMVgp Sma I (Part B) (or pCMV gp Nhe). (The *Cla*I site at position 5114 is methylated and not cut with *Cla*I). The product of that ligation is digested with *Nar*I, and the MLV-K *Nar*I fragment (positions 1035 to 1378) is inserted (SEQ ID NO: 1). This construct is designated pCMVgp -X (Figure 14).

EXAMPLE 4

CONSTRUCTION OF *ENV* EXPRESSION CASSETTES

A. Creation of a New 5' *Eag*I Restriction Site

Starting with an *Eag*I- *Eco*RI 626 bp subfragment from a 4070A amphotropic envelope (Chattopodhyay et al., *J. Vir. 39*:777, 1981; GenBank accession # MLV4070A, and #MLVENVC; SEQ ID NO: 12) cloned in a pBluescript II Ks+ vector (containing the start codon), site directed mutagenesis is performed upstream of the translation start site in order to change ACCATCCTCTGGACGGACATG... (SEQ ID NO: 7; positions 20 - 40 of Genebank sequence #MLVENVC) to ACCCGGCCGTGGACGGACATG... (SEQ ID NO: 8) and create a new *Eag*I site at position 23. This modification allows cloning of the amphotropic envelope sequence into an expression vector eliminating upstream 4070A sequence homologous to the *gag-pol* expression element as described in Example 2A.

B. Creation of a New 3' End for Env

A new 3' end of the envelope expression element is created by terminating the sequence which encodes the R-peptide downstream from the end of the transmembrane region (p15E). Briefly, construct pHCMV-PA, described above, is first modified by digestion with *Not*I (position 1097), blunted and relegated to obliterate the overlapping Bluescript *Eag*I site at the same position. pCMV Envam-Eag-X-less is then constructed by digesting the modified pHCMV-PA with *Eag*I (position 671 and *Sma*I (position 712) and ligating in two fragments. The first is an *EagI-NcoI* fragment from 4070A (positions 1-1455) (SEQ ID NO: 12). The second is an MLV-K envelope fragment, *NcoI - Pvu*II (positions 7227-7747) (SEQ ID NO: 12). The resultant construct from the three-way ligation contains the HCMV promoter followed by the SU (GP70) coding sequence of the 4070A envelope, the TM (p15e) coding sequence of MoMLV, and sequence encoding 8 residues of the R-peptide. In addition, this envelope expression cassette (pCMV Env am-Eag-X-less) (Figure 18) shares no sequence with crossless retrovector backbones described in Example 1.

C. Envelope Expression Element

Parts A and B from above are combined to complete an amphotropic expression element containing the CMV promoter, 4070A SU, MoMLV TM and SV40 polyadenylation signal in a Bluescript SK- plasmid vector. This construct is called pCMVenv-X (Figure 15). Briefly, the construct described in part A with a new *Eag*I restriction site is digested with *Eag*I and *Xho*I, and a 571 bp fragment is isolated. pCMV Envam-Eag-X-less (from part B) is digested with *Kpn*I and *Eag*I and the 695 bp fragment is reserved. pCMV Envam-Eag-X-less (from part B) is digested with *Kpn*I and *Xho*I and the 4649 bp fragment is reserved. These two fragments are ligated together along with the 571 bp *Eag*I to *Xho*I fragment digested from the PCR construct from part A. pCMVenv-X shares no sequence with crossless retrovector backbones nor the *gag-pol* expression element pCMVgp-X.

EXAMPLE 5

FUNCTIONALITY TESTS FOR *GAG-POL* AND *ENV* EXPRESSION CASSETTES

Rapid tests have been developed in order to ensure that the *gag-pol* and *env* expression cassettes are biologically active. The materials for these tests consist of a cell line used for transient expression (typically 293 cells, ATCC #CRL 1573), a target

cell line sensitive to infection (typically HT 1080 cells, ATCC #CCL 121) and either pRgpNeo (Figure 16) or pLARNL (Emi et al., *J. Virol 65*:1202-1207, 1991). The two later plasmids express rescuable retrovectors that confer G418 resistance and also express *gag-pol*, in the case of RgpNeo or *env*, in the case of pLARNL. For convenience, the organization of RgpNeo (Figure 16) is set forth below.

In order to test expression cassettes such as pCMVgp-X for functionality of *gag/pol*, the plasmid is co-transfected with pLARNL at a 1:1 ratio into 293 cells. After 12 hours, the media is replaced with normal growth media. After an additional 24 hours, supernatant fluid is removed from the 293 cells, filtered through a 0.45 μm filter, and placed on HT 1080 target cells. Twenty-four hours after that treatment, the media is replaced with growth media containing 800 ug/ml G418. G418 resistant colonies are scored after one week. The positive appearance of colonies indicates that all elements are functional and active in the original co-transfection.

For convenience, the organization of RgpNeo (Figure 16) is set forth below:
Position 1 = left end of 5' LTR; Positions 1-6320 = MoMLV sequence from 5'LTR to Sca 1 restriction site; Positions 6321 - 6675 = SV40 early promoter; Positions 6676-8001 = Neo resistance gene from Tn 5 (including prokaryotic promoter); and Positions 8002 - 8606 = pBR origin of replication.

EXAMPLE 6

PACKAGING CELL LINE AND PRODUCER CELL LINE DEVELOPMENT

This example describes the production of packing and producer cell lines utilizing the above described retroviral vector constructs, *gag/pol* expression cassettes, and *env* expression cassettes, which preclude the formation of replication competent virus.

Briefly, for amphotropic MoMLV-based retroviral vector constructs, a parent cell line is selected which lacks sequences which are homologous to Murine Leukemia Viruses, such as the dog cell line D-17 (ATCC No. CCL 183). The *gag/pol* expression cassettes are then introduced into the cell by electroporation, along with a selectable marker plasmid such as DHFR (Simonsen et al., *PNAS 80*:2495-2499, 1983). Resistant colonies are then selected, expanded in 6 well plates to confluency, and assayed for expression of gag/pol by Western Blots. Clones are also screened for the production of high titer vector particles after transduction with pLARNL.

The highest titer clones are then electroporated with an *env* expression cassette and a selectable marker plasmid such as hygromycin (*see* Gritz and Davies, *Gene 25*:179-188, 1983). Resistant colonies are selected, expanded in 6 well plates to confluency, and assayed for expression of env by Western Blots. Clones are also screened for the production of high titer vector particles after transduction with a retroviral vector construct.

Resultant packaging cell lines may be stored in liquid Nitrogen at 10 x 10^6 cells per vial, in DMEM containing 10% irradiated Fetal Bovine Serum, and 8% DMSO. Further testing may be accomplished in order to confirm sterility, and lack of helper virus production. Preferably, both an S+L- assay and a *Mus dunni* marker rescue assay should be performed in order to confirm a lack of helper virus production.

In order to construct a producer cell line, retroviral vector construct as described above in Example 1 is electroporated into a xenotropic packaging cell line made utilizing the methods described above. After 24-48 hours, supernatant fluid is removed from the xenotropic packaging cell line, and utilized to transduce a second packaging cell line, thereby creating the final producer cell line.

EXAMPLE 7

HELPER DETECTION ASSAY COCULTIVATION, AND MARKER RESCUE

This example describes a sensitive assay for the detection of replication competent retrovirus ("RCR"). Briefly, 5 x 10^5 vector-producing cells are cocultivated with an equal number of *Mus dunni* cells (Lander and Chattopadhyay, *J. Virol. 52*:695, 1984). *Mus dunni* cells are particularly preferred for helper virus detection because they are sensitive to nearly all murine leukemia-related viruses, and contain no known endogenous viruses. At three, six, and nine days after the initial culture, the cells are split approximately 1 to 10, and 5 x 10^5 fresh *Mus dunni* cells are added. Fifteen days after the initial cocultivation of *Mus dunni* cells with the vector-producing cells, supernatant fluid is removed from cultures, filtered through a 0.45 μm filter, and subjected to a marker rescue assay.

Briefly, culture fluid is removed from a MdH tester cell line (*Mus dunni* cells containing pLHL (a hygromycin resistance marker retroviral vector; *see* Palmer et al., *PNAS 84*(4):1055-1059, 1987) and replaced with the culture fluid to be tested. Polybrene is added to a final concentration of 4 μg/ml. On day 2, medium is removed and replaced with 2 ml of fresh DMEM containing 10% Fetal Calf Serum. On day 3, supernatant fluid is removed, filtered, and transferred to HT1080 cells. Polybrene is

added to a final concentration of 4μg/ml. On day 4, medium in the HT1080 cells is replaced with fresh DMEM containing 10% Fetal Calf Serum, and 100 μg/ml hygromycin. Selection is continued on days 5 through 20 until hygromycin resistant colonies can be scored, and all negative controls (*e.g.*, mock infected MdH cells) are dead.

EXAMPLE 8

ASSAY FOR ENCAPSIDATION OF WOBBLE RNA SEQUENCE

This example describes a sensitive assay for the detection of encapsidation of RNA from constructs containing wobble or normal gag sequence. Briefly, a fragment of DNA from a "wobble" *gag/pol* expression cassette (Example 3), containing the CMV promoter and gag sequence to the *Xho*I site (MoMLV position 1561) is ligated to a SV40 neo-3' LTR DNA fragment from N2 (Armentano et al., *supra*) or KT-3 (*see* WO 91/02805 or WO 92/05266). This construct is diagrammatically illustrated in Figure 19A, and is not expected to be encapsidated in packaging cell lines such as DA or HX (*see* WO 92/05266) because it lacks a 5' LTR and primer binding site.

A second construct is also made, similar to the first except that the wobble sequence is replaced by normal *gag* sequence. Similar to the first construct, the RNA transcribed from this DNA is not expected to be encapsidated. This construct is diagrammatically illustrated in Figure 19B.

The above constructs are separately transfected into a packaging cell line. The culture is then assayed for the ability to generate transducible G418-resistant retrovector. Neither construct results in transducible vector.

Cell cultures containing the above constructs are then transduced with the retrovector LHL (*see* Example 7). The cell cultures, after selection, will now generate retrovector conferring hygromycin resistance to target cells. Further, if co-encapsidation is allowed by interaction between LHL RNA and the transcripts from the above constructs, statistically significant RT-mediated recombination can occur resulting in the transfer of G418 resistance to target cells.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration,

various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Viagene, Inc.

(ii) TITLE OF INVENTION: CROSSLESS RETROVIRAL VECTORS

(iii) NUMBER OF SEQUENCES: 26

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Seed & Berry
(B) STREET: 6300 Columbia Center; 701 Fifth Avenue
(C) CITY: Seattle
(D) STATE: Washington
(E) COUNTRY: USA
(F) ZIP: 98104

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: PCT/US95/05789
(B) FILING DATE: 09-MAY-1995
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: McMasters, David D
(B) REGISTRATION NUMBER: 33,963
(C) REFERENCE/DOCKET NUMBER: 930049.424PC

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (206)622-4900
(B) TELEFAX: (206)682-6031

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8332 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GCGCCAGTCC TCCGATTGAC TGAGTCGCCC GGGTACCCGT GTATCCAATA AACCCTCTTG 60

CAGTTGCATC CGACTTGTGG TCTCGCTGTT CCTTGGGAGG GTCTCCTCTG AGTGATTGAC 120

TACCCGTCAG CGGGGGTCTT TCATTTGGGG GCTCGTCCGG GATCGGGAGA CCCCTGCCCA 180

GGGACCACCG ACCCACCACC GGGAGGTAAG CTGGCCAGCA ACTTATCTGT GTCTGTCCGA 240

TTGTCTAGTG TCTATGACTG ATTTTATGCG CCTGCGTCGG TACTAGTTAG CTAACTAGCT 300

CTGTATCTGG CGGACCCGTG GTGGAACTGA CGAGTTCGGA ACACCCGGCC GCAACCCTGG 360

GAGACGTCCC AGGGACTTCG GGGGCCGTTT TTGTGGCCCG ACCTGAGTCC AAAAATCCCG 420

ATCGTTTTGG ACTCTTTGGT GCACCCCCCT TAGAGGAGGG ATATGTGGTT CTGGTAGGAG 480

ACGAGAACCT AAAACAGTTC CCGCCTCCGT CTGAATTTTT GCTTTCGGTT TGGGACCGAA 540

GCCGCGCCGC GCGTCTTGTC TGCTGCAGCA TCGTTCTGTG TTGTCTCTGT CTGACTGTGT 600

TTCTGTATTT GTCTGAGAAT ATGGGCCAGA CTGTTACCAC TCCCTTAAGT TTGACCTTAG 660

GTCACTGGAA AGATGTCGAG CGGATCGCTC ACAACCAGTC GGTAGATGTC AAGAAGAGAC 720

GTTGGGTTAC CTTCTGCTCT GCAGAATGGC CAACCTTTAA CGTCGGATGG CCGCGAGACG 780

GCACCTTTAA CCGAGACCTC ATCACCCAGG TTAAGATCAA GGTCTTTTCA CCTGGCCGC 840

ATGGACACCC AGACCAGGTC CCCTACATCG TGACCTGGGA AGCCTTGGCT TTTGACCCCC 900

CTCCCTGGGT CAAGCCCTTT GTACACCCTA AGCCTCCGCC TCCTCTTCCT CCATCCGCCC 960

CGTCTCTCCC CCTTGAACCT CCTCGTTCGA CCCCGCCTCG ATCCTCCCTT TATCCAGCCC 1020

TCACTCCTTC TCTAGGCGCC AAACCTAAAC CTCAAGTTCT TTCTGACAGT GGGGGGCCGC 1080

TCATCGACCT ACTTACAGAA GACCCCCCGC CTTATAGGGA CCCAAGACCA CCCCCTTCCG 1140

ACAGGGACGG AAATGGTGGA GAAGCGACCC CTGCGGGAGA GGCACCGGAC CCCTCCCCAA 1200

TGGCATCTCG CCTACGTGGG AGACGGGAGC CCCCTGTGGC CGACTCCACT ACCTCGCAGG 1260

CATTCCCCCT CCGCGCAGGA GGAAACGGAC AGCTTCAATA CTGGCCGTTC TCCTCTTCTG 1320

ACCTTTACAA CTGGAAAAAT AATAACCCTT CTTTTTCTGA AGATCCAGGT AAACTGACAG 1380

CTCTGATCGA GTCTGTTCTC ATCACCCATC AGCCCACCTG GGACGACTGT CAGCAGCTGT 1440

TGGGGACTCT GCTGACCGGA GAAGAAAAAC AACGGGTGCT CTTAGAGGCT AGAAAGGCGG 1500

TGCGGGGCGA TGATGGGCGC CCCACTCAAC TGCCCAATGA AGTCGATGCC GCTTTTCCCC 1560

TCGAGCGCCC AGACTGGGAT TACACCACCC AGGCAGGTAG GAACCACCTA GTCCACTATC 1620

GCCAGTTGCT CCTAGCGGGT CTCCAAAACG CGGGCAGAAG CCCCACCAAT TTGGCCAAGG 1680

TAAAAGGAAT AACACAAGGG CCCAATGAGT CTCCCTCGGC CTTCCTAGAG AGACTTAAGG 1740

AAGCCTATCG CAGGTACACT CCTTATGACC CTGAGGACCC AGGGCAAGAA ACTAATGTGT 1800

CTATGTCTTT CATTTGGCAG TCTGCCCCAG ACATTGGGAG AAAGTTAGAG AGGTTAGAAG 1860

ATTTAAAAAA CAAGACGCTT GGAGATTTGG TTAGAGAGGC AGAAAAGATC TTTAATAAAC 1920

GAGAAACCCC GGAAGAAAGA GAGGAACGTA TCAGGAGAGA AACAGAGGAA AAAGAAGAAC 1980

GCCGTAGGAC AGAGGATGAG CAGAAAGAGA AAGAAAGAGA TCGTAGGAGA CATAGAGAGA 2040

TGAGCAAGCT ATTGGCCACT GTCGTTAGTG GACAGAAACA GGATAGACAG GGAGGAGAAC 2100

GAAGGAGGTC CCAACTCGAT CGCGACCAGT GTGCCTACTG CAAAGAAAAG GGGCACTGGG 2160

CTAAAGATTG TCCCAAGAAA CCACGAGGAC CTCGGGGACC AAGACCCCAG ACCTCCCTCC 2220

TGACCCTAGA TGACTAGGGA GGTCAGGGTC AGGAGCCCCC CCCTGAACCC AGGATAACCC 2280

TCAAAGTCGG GGGGCAACCC GTCACCTTCC TGGTAGATAC TGGGGCCCAA CACTCCGTGC 2340

TGACCCAAAA TCCTGGACCC CTAAGTGATA AGTCTGCCTG GGTCCAAGGG GCTACTGGAG 2400

GAAAGCGGTA TCGCTGGACC ACGGATCGCA AAGTACATCT AGCTACCGGT AAGGTCACCC 2460

ACTCTTTCCT CCATGTACCA GACTGTCCCT ATCCTCTGTT AGGAAGAGAT TTGCTGACTA 2520

AACTAAAAGC CCAAATCCAC TTTGAGGGAT CAGGAGCTCA GGTTATGGGA CCAATGGGGC 2580

AGCCCCTGCA AGTGTTGACC CTAAATATAG AAGATGAGCA TCGGCTACAT GAGACCTCAA 2640

AAGAGCCAGA TGTTTCTCTA GGGTCCACAT GGCTGTCTGA TTTTCCTCAG GCCTGGGCGG 2700

AAACCGGGGG CATGGGACTG GCAGTTCGCC AAGCTCCTCT GATCATACCT CTGAAAGCAA 2760

CCTCTACCCC CGTGTCCATA AAACAATACC CCATGTCACA AGAAGCCAGA CTGGGGATCA 2820

AGCCCCACAT ACAGAGACTG TTGGACCAGG GAATACTGGT ACCCTGCCAG TCCCCCTGGA 2880

ACACGCCCCT GCTACCCGTT AAGAAACCAG GGACTAATGA TTATAGGCCT GTCCAGGATC 2940

TGAGAGAAGT CAACAAGCGG GTGGAAGACA TCCACCCCAC CGTGCCCAAC CCTTACAACC 3000

TCTTGAGCGG GCTCCCACCG TCCCACCAGT GGTACACTGT GCTTGATTTA AAGGATGCCT 3060

TTTTCTGCCT GAGACTCCAC CCCACCAGTC AGCCTCTCTT CGCCTTTGAG TGGAGAGATC 3120

CAGAGATGGG AATCTCAGGA CAATTGACCT GGACCAGACT CCCACAGGGT TTCAAAAACA 3180

GTCCCACCCT GTTTGATGAG GCACTGCACA GAGACCTAGC AGACTTCCGG ATCCAGCACC 3240

CAGACTTGAT CCTGCTACAG TACGTGGATG ACTTACTGCT GGCCGCCACT TCTGAGCTAG 3300

ACTGCCAACA AGGTACTCGG GCCCTGTTAC AAACCCTAGG GAACCTCGGG TATCGGGCCT 3360

CGGCCAAGAA AGCCCAAATT TGCCAGAAAC AGGTCAAGTA TCTGGGGTAT CTTCTAAAAG 3420

AGGGTCAGAG ATGGCTGACT GAGGCCAGAA AAGAGACTGT GATGGGGCAG CCTACTCCGA 3480

AGACCCCTCG ACAACTAAGG GAGTTCCTAG GGACGGCAGG CTTCTGTCGC CTCTGGATCC 3540

CTGGGTTTGC AGAAATGGCA GCCCCCTTGT ACCCTCTCAC CAAAACGGGG ACTCTGTTTA 3600

ATTGGGGCCC AGACCAACAA AAGGCCTATC AAGAAATCAA GCAAGCTCTT CTAACTGCCC 3660

CAGCCCTGGG GTTGCCAGAT TTGACTAAGC CCTTTGAACT CTTTGTCGAC GAGAAGCAGG 3720

GCTACGCCAA AGGTGTCCTA ACGCAAAAAC TGGGACCTTG GCGTCGGCCG GTGGCCTACC 3780

TGTCCAAAAA GCTAGACCCA GTAGCAGCTG GGTGGCCCCC TTGCCTACGG ATGGTAGCAG 3840

CCATTGCCGT ACTGACAAAG GATGCAGGCA AGCTAACCAT GGGACAGCCA CTAGTCATTC 3900

TGGCCCCCCA TGCAGTAGAG GCACTAGTCA AACAACCCCC CGACCGCTGG CTTTCCAACG 3960

CCCGGATGAC TCACTATCAG GCCTTGCTTT TGGACACGGA CCGGGTCCAG TTCGGACCGG 4020

TGGTAGCCCT GAACCCGGCT ACGCTGCTCC CACTGCCTGA GGAAGGGCTG CAACACAACT 4080

GCCTTGATAT CCTGGCCGAA GCCCACGGAA CCCGACCCGA CCTAACGGAC CAGCCGCTCC 4140

CAGACGCCGA CCACACCTGG TACACGGATG GAAGCAGTCT CTTACAAGAG GGACAGCGTA 4200

AGGCGGGAGC TGCGGTGACC ACCGAGACCG AGGTAATCTG GGCTAAAGCC CTGCCAGCCG 4260

GGACATCCGC TCAGCGGGCT GAACTGATAG CACTCACCCA GGCCCTAAAG ATGGCAGAAG 4320

GTAAGAAGCT AAATGTTTAT ACTGATAGCC GTTATGCTTT TGCTACTGCC CATATCCATG 4380

GAGAAATATA CAGAAGGCGT GGGTTGCTCA CATCAGAAGG CAAAGAGATC AAAAATAAAG 4440

ACGAGATCTT GGCCCTACTA AAAGCCCTCT TTCTGCCCAA AAGACTTAGC ATAATCCATT 4500

GTCCAGGACA TCAAAAGGGA CACAGCGCCG AGGCTAGAGG CAACCGGATG GCTGACCAAG 4560

CGGCCCGAAA GGCAGCCATC ACAGAGACTC CAGACACCTC TACCCTCCTC ATAGAAAATT 4620

CATCACCCTA CACCTCAGAA CATTTTCATT ACACAGTGAC TGATATAAAG GACCTAACCA 4680

AGTTGGGGGC CATTTATGAT AAAACAAAGA AGTATTGGGT CTACCAAGGA AAACCTGTGA 4740

TGCCTGACCA GTTTACTTTT GAATTATTAG ACTTTCTTCA TCAGCTGACT CACCTCAGCT 4800

TCTCAAAAAT GAAGGCTCTC CTAGAGAGAA GCCACAGTCC CTACTACATG CTGAACCGGG 4860

ATCGAACACT CAAAAATATC ACTGAGACCT GCAAAGCTTG TGCACAAGTC AACGCCAGCA 4920

AGTCTGCCGT TAAACAGGGA ACTAGGGTCC GCGGGCATCG GCCCGGCACT CATTGGGAGA 4980

TCGATTTCAC CGAGATAAAG CCCGGATTGT ATGGCTATAA ATATCTTCTA GTTTTTATAG 5040

ATACCTTTTC TGGCTGGATA GAAGCCTTCC CAACCAAGAA AGAAACCGCC AAGGTCGTAA 5100

CCAAGAAGCT ACTAGAGGAG ATCTTCCCCA GGTTCGGCAT GCCTCAGGTA TTGGGAACTG 5160

ACAATGGGCC TGCCTTCGTC TCCAAGGTGA GTCAGACAGT GGCCGATCTG TTGGGGATTG 5220

ATTGGAAATT ACATTGTGCA TACAGACCCC AAAGCTCAGG CCAGGTAGAA AGAATGAATA 5280

GAACCATCAA GGAGACTTTA ACTAAATTAA CGCTTGCAAC TGGCTCTAGA GACTGGGTGC 5340

TCCTACTCCC CTTAGCCCTG TACCGAGCCC GCAACACGCC GGGCCCCCAT GGCCTCACCC 5400

CATATGAGAT CTTATATGGG GCACCCCCGC CCCTTGTAAA CTTCCCTGAC CCTGACATGA 5460

CAAGAGTTAC TAACAGCCCC TCTCTCCAAG CTCACTTACA GGCTCTCTAC TTAGTCCAGC 5520

ACGAAGTCTG GAGACCTCTG GCGGCAGCCT ACCAAGAACA ACTGGACCGA CCGGTGGTAC 5580

CTCACCCTTA CCGAGTCGGC GACACAGTGT GGGTCCGCCG ACACCAGACT AAGAACCTAG 5640

AACCTCGCTG GAAAGGACCT TACACAGTCC TGCTGACCAC CCCCACCGCC CTCAAAGTAG 5700

ACGGCATCGC AGCTTGGATA CACGCCGCCC ACGTGAAGGC TGCCGACCCC GGGGGTGGAC 5760

CATCCTCTAG ACTGACATGG CGCGTTCAAC GCTCTCAAAA CCCCTTAAAA ATAAGGTTAA 5820

CCCGCGAGGC CCCCTAATCC CCTTAATTCT TCTGATGCTC AGAGGGGTCA GTACTGCTTC 5880

GCCCGGCTCC AGTCCTCATC AAGTCTATAA TATCACCTGG GAGGTAACCA ATGGAGATCG 5940

GGAGACGGTA TGGGCAACTT CTGGCAACCA CCCTCTGTGG ACCTGGTGGC CTGACCTTAC 6000

CCCAGATTTA TGTATGTTAG CCCACCATGG ACCATCTTAT TGGGGGCTAG AATATCAATC 6060

CCCTTTTTCT TCTCCCCCGG GGCCCCCTTG TTGCTCAGGG GGCAGCAGCC CAGGCTGTTC 6120

CAGAGACTGC GAAGAACCTT TAACCTCCCT CACCCCTCGG TGCAACACTG CCTGGAACAG 6180

ACTCAAGCTA GACCAGACAA CTCATAAATC AAATGAGGGA TTTTATGTTT GCCCCGGGCC 6240

CCACCGCCCC CGAGAATCCA AGTCATGTGG GGGTCCAGAC TCCTTCTACT GTGCCTATTG 6300

GGGCTGTGAG ACAACCGGTA GAGCTTACTG GAAGCCCTCC TCATCATGGG ATTTCATCAC 6360

AGTAAACAAC AATCTCACCT CTGACCAGGC TGTCCAGGTA TGCAAAGATA ATAAGTGGTG 6420

CAACCCCTTA GTTATTCGGT TTACAGACGC CGGGAGACGG GTTACTTCCT GGACCACAGG 6480

ACATTACTGG GGCTTACGTT TGTATGTCTC CGGACAAGAT CCAGGGCTTA CATTTGGGAT 6540

CCGACTCAGA TACCAAAATC TAGGACCCCG CGTCCCAATA GGGCCAAACC CCGTTCTGGC 6600

AGACCAACAG CCACTCTCCA AGCCCAAACC TGTTAAGTCG CCTTCAGTCA CCAAACCACC 6660

CAGTGGGACT CCTCTCTCCC CTACCCAACT TCCACCGGCG GGAACGGAAA ATAGGCTGCT 6720

AAACTTAGTA GACGGAGCCT ACCAAGCCCT CAACCTCACC AGTCCTGACA AAACCCAAGA 6780

GTGCTGGTTG TGTCTAGTAG CGGGACCCCC CTACTACGAA GGGGTTGCCG TCCTGGGTAC 6840

CTACTCCAAC CATACCTCTG CTCCAGCCAA CTGCTCCGTG GCCTCCCAAC ACAAGTTGAC 6900

CCTGTCCGAA GTGACCGGAC AGGGACTCTG CATAGGAGCA GTTCCCAAAA CACATCAGGC 6960

CCTATGTAAT ACCACCCAGA CAAGCAGTCG AGGGTCCTAT TATCTAGTTG CCCCTACAGG 7020

TACCATGTGG GCTTGTAGTA CCGGGCTTAC TCCATGCATC TCCACCACCA TACTGAACCT 7080

TACCACTGAT TATTGTGTTC TTGTCGAACT CTGGCCAAGA GTCACCTATC ATTCCCCCAG 7140

CTATGTTTAC GGCCTGTTTG AGAGATCCAA CCGACACAAA AGAGAACCGG TGTCGTTAAC 7200

CCTGGCCCTA TTATTGGGTG GACTAACCAT GGGGGGAATT GCCGCTGGAA TAGGAACAGG 7260

GACTACTGCT CTAATGGCCA CTCAGCAATT CCAGCAGCTC CAAGCCGCAG TACAGGATGA 7320

TCTCAGGGAG GTTGAAAAAT CAATCTCTAA CCTAGAAAAG TCTCTCACTT CCCTGTCTGA 7380

AGTTGTCCTA CAGAATCGAA GGGGCCTAGA CTTGTTATTT CTAAAAGAAG GAGGGCTGTG 7440

TGCTGCTCTA AAAGAAGAAT GTTGCTTCTA TGCGGACCAC ACAGGACTAG TGAGAGACAG 7500

CATGGCCAAA TTGAGAGAGA GGCTTAATCA GAGACAGAAA CTGTTTGAGT CAACTCAAGG 7560

ATGGTTTGAG GGACTGTTTA ACAGATCCCC TTGGTTTACC ACCTTGATAT CTACCATTAT 7620

GGGACCCCTC ATTGTACTCC TAATGATTTT GCTCTTCGGA CCCTGCATTC TTAATCGATT 7680

AGTCCAATTT GTTAAAGACA GGATATCAGT GGTCCAGGCT CTAGTTTTGA CTCAACAATA 7740

TCACCAGCTG AAGCCTATAG AGTACGAGCC ATAGATAAAA TAAAAGATTT TATTTAGTCT 7800

CCAGAAAAAG GGGGGAATGA AAGACCCCAC CTGTAGGTTT GGCAAGCTAG CTTAAGTAAC 7860

GCCATTTTGC AAGGCATGGA AAAATACATA ACTGAGAATA GAGAAGTTCA GATCAAGGTC 7920

AGGAACAGAT GGAACAGCTG AATATGGGCC AAACAGGATA TCTGTGGTAA GCAGTTCCTG 7980

CCCCGGCTCA GGGCCAAGAA CAGATGGAAC AGCTGAATAT GGGCCAAACA GGATATCTGT 8040

GGTAAGCAGT TCCTGCCCCG GCTCAGGGCC AAGAACAGAT GGTCCCCAGA TGCGGTCCAG 8100

CCCTCAGCAG TTTCTAGAGA ACCATCAGAT GTTTCCAGGG TGCCCCAAGG ACCTGAAATG 8160

ACCCTGTGCC TTATTTGAAC TAACCAATCA GTTCGCTTCT CGCTTCTGTT CGCGCGCTTC 8220

TGCTCCCCGA GCTCAATAAA AGAGCCCACA ACCCCTCACT CGGGGCGCCA GTCCTCCGAT 8280

TGACTGAGTC GCCCGGGTAC CCGTGTATCC AATAAACCCT CTTGCAGTTG CA 8332

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE OESCRIPTION: SEQ IO NO:2:

GGTAACAGTC TGGCCCGAAT TCTCAGACAA ATACAG 36

2) INFORMATION FOR SEQ IO NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEONESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CTGTATTTGT CTGAGAATTA AGGCTAGACT GTTACCAC 38

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE OESCRIPTION: SEQ ID NO:4:

ATATATATAT ATCGATACCA TG 22

(2) INFORMATION FOR SEQ IO NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANOEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE OESCRIPTION: SEQ ID NO:5:

GGCGCCAAAC CTAAAC 16

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Ser Lys Asn Tyr Pro 5

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ACCATCCTCT GGACGGACAT G 21

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ACCCGGCCGT GGACGGACAT G 21

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 449 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 20..442

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
ATATATATAT ATCGATACC ATG GGG CAA ACC GTG ACT ACC CCT CTG TCC CTC      52
                     Met Gly Gln Thr Val Thr Thr Pro Leu Ser Leu
                       1               5                  10

ACA CTG GGC CAT TGG AAG GAC GTG GAA AGA ATT GCC CAT AAT CAA AGC     100
Thr Leu Gly His Trp Lys Asp Val Glu Arg Ile Ala His Asn Gln Ser
            15                  20                  25

GTG GAC GTC AAA AAA CGC AGG TGG GTG ACA TTT TGT AGC GCC GAG TGG     148
Val Asp Val Lys Lys Arg Arg Trp Val Thr Phe Cys Ser Ala Glu Trp
        30                  35                  40

CCC ACA TTC AAT GTT GGC TGG CCT AGG GAT GGA ACT TTC AAT CGC GAT     196
Pro Thr Phe Asn Val Gly Trp Pro Arg Asp Gly Thr Phe Asn Arg Asp
    45                  50                  55

CTG ATT ACT CAA GTG AAA ATT AAA GTG TTC AGC CCC GGA CCC CAC GGC     244
Leu Ile Thr Gln Val Lys Ile Lys Val Phe Ser Pro Gly Pro His Gly
60                  65                  70                  75

CAT CCC GAT CAA GTT CCT TAT ATT GTC ACA TGG GAG GCT CTC GCT TTC     292
His Pro Asp Gln Val Pro Tyr Ile Val Thr Trp Glu Ala Leu Ala Phe
                80                  85                  90

GAT CCA CCA CCT TGG GTG AAA CCA TTC GTG CAT CCC AAA CCA CCT CCA     340
Asp Pro Pro Pro Trp Val Lys Pro Phe Val His Pro Lys Pro Pro Pro
            95                  100                 105

CCC CTC CCA CCC AGC GCT CCT AGC CTG CCC TTG GAG CCC CCA CGA AGC     388
Pro Leu Pro Pro Ser Ala Pro Ser Leu Pro Leu Glu Pro Pro Arg Ser
        110                 115                 120

ACA CCA CCC AGG AGC AGC TTG TAC CCT GCT CTG ACC CCC AGC CTC GGC     436
Thr Pro Pro Arg Ser Ser Leu Tyr Pro Ala Leu Thr Pro Ser Leu Gly
    125                 130                 135

GCC AAA CCTAAAC                                                    449
Ala Lys
140
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 141 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Met Gly Gln Thr Val Thr Thr Pro Leu Ser Leu Thr Leu Gly His Trp
 1               5                  10                  15
```

Lys Asp Val Glu Arg Ile Ala His Asn Gln Ser Val Asp Val Lys Lys
20 25 30

```
Arg Arg Trp Val Thr Phe Cys Ser Ala Glu Trp Pro Thr Phe Asn Val
        35                  40                  45

Gly Trp Pro Arg Asp Gly Thr Phe Asn Arg Asp Leu Ile Thr Gln Val
    50                  55                  60

Lys Ile Lys Val Phe Ser Pro Gly Pro His Gly His Pro Asp Gln Val
65                  70                  75                  80

Pro Tyr Ile Val Thr Trp Glu Ala Leu Ala Phe Asp Pro Pro Pro Trp
                85                  90                  95

Val Lys Pro Phe Val His Pro Lys Pro Pro Pro Pro Leu Pro Pro Ser
            100                 105                 110

Ala Pro Ser Leu Pro Leu Glu Pro Pro Arg Ser Thr Pro Pro Arg Ser
        115                 120                 125

Ser Leu Tyr Pro Ala Leu Thr Pro Ser Leu Gly Ala Lys
    130                 135                 140
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 420 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..420

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
ATG GGC CAG ACT GTT ACC ACT CCC TTA AGT TTG ACC TTA GGT CAC TGG      48
Met Gly Gln Thr Val Thr Thr Pro Leu Ser Leu Thr Leu Gly His Trp
 1               5                  10                  15

AAA GAT GTC GAG CGG ATC GCT CAC AAC CAG TCG GTA GAT GTC AAG AAG      96
Lys Asp Val Glu Arg Ile Ala His Asn Gln Ser Val Asp Val Lys Lys
            20                  25                  30

AGA CGT TGG GTT ACC TTC TGC TCT GCA GAA TGG CCA ACC TTT AAC GTC     144
Arg Arg Trp Val Thr Phe Cys Ser Ala Glu Trp Pro Thr Phe Asn Val
        35                  40                  45

GGA TGG CCG CGA GAC GGC ACC TTT AAC CGA GAC CTC ATC ACC CAG GTT     192
Gly Trp Pro Arg Asp Gly Thr Phe Asn Arg Asp Leu Ile Thr Gln Val
    50                  55                  60

AAG ATC AAG GTC TTT TCA CCT GGC CCG CAT GGA CAC CCA GAC CAG GTC     240
Lys Ile Lys Val Phe Ser Pro Gly Pro His Gly His Pro Asp Gln Val
65                  70                  75                  80
```

```
CCC TAC ATC GTG ACC TGG GAA GCC TTG GCT TTT GAC CCC CCT CCC TGG      288
Pro Tyr Ile Val Thr Trp Glu Ala Leu Ala Phe Asp Pro Pro Pro Trp
                85                  90                  95

GTC AAG CCC TTT GTA CAC CCT AAG CCT CCG CCT CCT CTT CCT CCA TCC      336
Val Lys Pro Phe Val His Pro Lys Pro Pro Pro Pro Leu Pro Pro Ser
            100                 105                 110

GCC CCG TCT CTC CCC CTT GAA CCT CCT CGT TCG ACC CCG CCT CGA TCC      384
Ala Pro Ser Leu Pro Leu Glu Pro Pro Arg Ser Thr Pro Pro Arg Ser
        115                 120                 125

TCC CTT TAT CCA GCC CTC ACT CCT TCT CTA GGC GCC                      420
Ser Leu Tyr Pro Ala Leu Thr Pro Ser Leu Gly Ala
    130                 135                 140
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 140 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
Met Gly Gln Thr Val Thr Thr Pro Leu Ser Leu Thr Leu Gly His Trp
 1               5                  10                  15

Lys Asp Val Glu Arg Ile Ala His Asn Gln Ser Val Asp Val Lys Lys
            20                  25                  30

Arg Arg Trp Val Thr Phe Cys Ser Ala Glu Trp Pro Thr Phe Asn Val
        35                  40                  45

Gly Trp Pro Arg Asp Gly Thr Phe Asn Arg Asp Leu Ile Thr Gln Val
    50                  55                  60

Lys Ile Lys Val Phe Ser Pro Gly Pro His Gly His Pro Asp Gln Val
65                  70                  75                  80

Pro Tyr Ile Val Thr Trp Glu Ala Leu Ala Phe Asp Pro Pro Pro Trp
                85                  90                  95

Val Lys Pro Phe Val His Pro Lys Pro Pro Pro Pro Leu Pro Pro Ser
            100                 105                 110

Ala Pro Ser Leu Pro Leu Glu Pro Pro Arg Ser Thr Pro Pro Arg Ser
        115                 120                 125

Ser Leu Tyr Pro Ala Leu Thr Pro Ser Leu Gly Ala
    130                 135                 140
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2001 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GGCCGACACC CAGAGTGGAC CATCCTCTGG ACGGACATGG CGCGTTCAAC GCTCTCAAAA 60

CCCCCTCAAG ATAAGATTAA CCCGTGGAAG CCCTTAATAG TCATGGGAGT CCTGTTAGGA 120

GTAGGGATGG CAGAGAGCCC CCATCAGGTC TTTAATGTAA CCTGGAGAGT CACCAACCTG 180

ATGACTGGGC GTACCGCCAA TGCCACCTCC CTCCTGGGAA CTGTACAAGA TGCCTTCCCA 240

AAATTATATT TTGATCTATG TGATCTGGTC GGAGAGGAGT GGGACCCTTC AGACCAGGAA 300

CCGTATGTCG GGTATGGCTG CAAGTACCCC GCAGGGAGAC AGCGGACCCG GACTTTTGAC 360

TTTTACGTGT GCCCTGGGCA TACCGTAAAG TCGGGGTGTG GGGGACCAGG AGAGGGCTAC 420

TGTGGTAAAT GGGGGTGTGA AACCACCGGA CAGGCTTACT GGAAGCCCAC ATCATCGTGG 480

GACCTAATCT CCCTTAAGCG CGGTAACACC CCCTGGGACA CGGGATGCTC TAAAGTTGCC 540

TGTGGCCCCT GCTACGACCT CTCCAAAGTA TCCAATTCCT TCCAAGGGGC TACTCGAGGG 600

GGCAGATGCA ACCCTCTAGT CCTAGAATTC ACTGATGCAG GAAAAAAGGC TAACTGGGAC 660

GGGCCCAAAT CGTGGGGACT GAGACTGTAC CGGACAGGAA CAGATCCTAT TACCATGTTC 720

TCCCTGACCC GGCAGGTCCT TAATGTGGGA CCCCGAGTCC CCATAGGGCC CAACCCAGTA 780

TTACCCGACC AAAGACTCCC TTCCTCACCA ATAGAGATTG TACCGGCTCC ACAGCCACCT 840

AGCCCCCTCA ATACCAGTTA CCCCCCTTCC ACTACCAGTA CACCCTCAAC CTCCCCTACA 900

AGTCCAAGTG TCCCACAGCC ACCCCCAGGA ACTGGAGATA GACTACTAGC TCTAGTCAAA 960

GGAGCCTATC AGGCGCTTAA CCTCACCAAT CCCGACAAGA CCCAAGAATG TTGGCTGTGC 1020

TTAGTGTCGG GACCTCCTTA TTACGAAGGA GTAGCGGTCG TGGGCACTTA TACCAATCAT 1080

TCCACCGCTC CGGCCAACTG TACGGCCACT TCCCAACATA AGCTTACCCT ATCTGAAGTG 1140

ACAGGACAGG GCCTATGCAT GGGGGCAGTA CCTAAAACTC ACCAGGCCTT ATGTAACACC 1200

ACCCAAAGCG CCGGCTCAGG ATCCTACTAC CTTGCAGCAC CCGCCGGAAC AATGTGGGCT 1260

TGCAGCACTG GATTGACTCC CTGCTTGTCC ACCACGGTGC TCAATCTAAC CACAGATTAT 1320

TGTGTATTAG TTGAACTCTG GCCCAGAGTA ATTTACCACT CCCCCGATTA TATGTATGGT 1380

CAGCTTGAAC AGCGTACCAA ATATAAAAGA GAGCCAGTAT CATTGACCCT GGCCCTTCTA 1440

CTAGGAGGAT TAACCATGGG AGGGATTGCA GCTGGAATAG GGACGGGGAC CACTGCCTTA 1500

ATTAAAACCC AGCAGTTTGA GCAGCTTCAT GCCGCTATCC AGACAGACCT CAACGAAGTC 1560

GAAAAGTCAA TTACCAACCT AGAAAAGTCA CTGACCTCGT TGTCTGAAGT AGTCCTACAG 1620

AACCGCAGAG GCCTAGATTT GCTATTCCTA AAGGAGGGAG GTCTCTGCGC AGCCCTAAAA 1680

GAAGAATGTT GTTTTTATGC AGACCACACG GGGCTAGTGA GAGACAGCAT GGCCAAATTA 1740

AGAGAAAGGC TTAATCAGAG ACAAAAACTA TTTGAGACAG GCCAAGGATG GTTCGAAGGG 1800

CTGTTTAATA GATCCCCCTG GTTTACCACC TTAATCTCCA CCATCATGGG ACCTCTAATA 1860

GTACTCTTAC TGATCTTACT CTTTGGACCT TGCATTCTCA ATCGATTGGT CCAATTTGTT 1920

AAAGACAGGA TCTCAGTGGT CCAGGCTCTG GTTTTGACTC AGCAATATCA CCAGCTAAAA 1980

CCCATAGAGT ACGAGCCATG A 2001

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CTAGCTAGCT AG 12

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 64 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

ATATATATAT ATCGATACCA TGGGGCAAAC CGTGACTACC CCTCTGTCCC TCACACTGGC 60

CCAA 64

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TTGATTATGG GCAATTCTTT CCACGTCCTT CCAATGGCCC AGTGTGAGGG AC 52

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 72 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

AGAATTGCCC ATAATCAAAG CGTGGACGTC AAAAAACGCA GGTGGGTGAC ATTTTGTAGC 60

GCCGAGTGGC CC 72

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

AAGTTCCATC CCTAGGCCAG CCAACATTGA ATGTGGGCCA CTCGGCGCTA CA 52

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 72 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GGCCTAGGGA TGGAACTTTC AATCGCGATC TGATTACTCA AGTGAAAATT AAAGTGTTCA 60

GCCCCGGACC CC 72

(2) INFORMATION FOR SEQ IO NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE OESCRIPTION: SEQ ID NO:20:

GTGACAATAT AAGGAACTTG ATCGGGATGG CCGTGGGGTC CGGGGCTGAA CA 52

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 72 base pairs
(B) TYPE: nucleic acid
(C) STRANOEONESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

AGTTCCTTAT ATTGTCACAT CGGAGGCTCT CGCTTTCGAT CCACCACCTT GGGTGAAACC 60

ATTCGTGCAT CC 72

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: nucleic acid
(C) STRANOEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22

AGGAGCGCTG GGTGGGAGGG GTGGAGGTGG TTTGGGATGC ACGAATGGTT TC 52

(2) INFORMATION FOR SEQ IO NO:23

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 72 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CTCCCACCCA GCGCTCCTAG CCTGCCCTTG GAGCCCCCAC GAAGCACACC ACCCAGGAGC 60

AGCTTGTACC CT 72

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GTTTAGGTTT GGCGCCGAGG CTGGGGGTCA GAGCAGGGTA CAAGCTGCTC CT 52

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

ATATATATAT ATCGATACC 19

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GTTTAGGTTT GGCGCCGAGG 20

Claims

1. A retroviral vector construct comprising a 5' LTR, a tRNA binding site, a packaging signal, one or more heterologous sequences, an origin of second strand DNA synthesis and a 3' LTR, wherein said vector construct lacks *gag/pol* and *env* coding sequences.

2. The retroviral vector construct according to claim 1 wherein said vector construct lacks an extended packaging signal.

3. The retroviral vector construct according to claim 1 wherein said construct lacks a retroviral nucleic acid sequence upstream of said 5' LTR.

4. The retroviral vector construct according to claim 3 wherein said construct lacks an *env* coding sequence upstream of said 5' LTR.

5. The retroviral vector construct according to claim 1 wherein said construct lacks a retroviral packaging signal sequence downstream of said 3' LTR.

6. The retroviral vector construct according to any one of claims 1 to 5 wherein said retrovector is constructed from a retrovirus selected from the group consisting of amphotropic, ecotropic, xenotropic or polytropic viruses.

7. The retroviral vector construct according to any one of the claims 1 to 5 wherein said retrovector is constructed from a Murine Leukemia Virus.

8. The retroviral vector construct according to any one of claims 1 to 5, wherein said heterologous sequence is at least x kb in length, wherein x is selected from the group consisting of 2, 3, 4, 5, 6, 7 and 8.

9. The retroviral vector construct according to claim 8 wherein said heterologous sequence is a gene encoding a cytotoxic protein.

10. The retroviral vector construct according to claim 9 wherein said cytotoxic protein is selected from the group consisting of ricin, abrin, diphtheria toxin, cholera

toxin, gelonin, pokeweed, antiviral protein, tritin, Shigella toxin, and Pseudomonas exotoxin A.

11. The retroviral vector construct according to claim 8 wherein said heterologous sequence is an antisense sequence.

12. The retroviral vector construct according to claim 8 wherein said heterologous sequence encodes an immune accessory molecule.

13. The retroviral vector construct according to claim 12 wherein said immune accessory molecule is selected from the group consisting of IL-1, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, and IL-13.

14. The retroviral vector construct according to claim 12 wherein said immune accessory molecule is selected from the group consisting of IL-2, IL-12, IL-15 and gamma-interferon.

15. The retroviral vector construct according to claim 12 wherein said immune accessory molecule is selected from the group consisting of ICAM-1, ICAM-2, β-microglobin, LFA3, HLA class I and HLA class II molecules.

16. The retroviral vector construct according to claim 8 wherein said heterologous sequence encodes a gene product that activates a compound with little or no cytotoxicity into a toxic product.

17. The retroviral vector construct according to claim 16 wherein said gene product is selected from the group consisting of HSVTK and VZVTK.

18. The retroviral vector construct according to claim 8 wherein said heterologous sequence is a ribozyme.

19. The retroviral vector construct according to claim 8 wherein said heterologous sequence is a replacement gene.

20. The retroviral vector construct according to claim 19 wherein said replacement gene encodes a protein selected from the group consisting of Factor VIII, ADA, HPRT, CF and the LDL Receptor.

21. The retroviral vector construct according to claim 8 wherein said heterologous sequence encodes an immunogenic portion of a virus selected from the group consisting of HBV, HCV, HPV, EBV, FeLV, FIV, and HIV.

22. A *gag/pol* expression cassette, comprising a promoter operably linked to a *gag/pol* gene, and a polyadenylation sequence, wherein said *gag/pol* gene has been modified to contain codons which are degenerate for gag.

23. The *gag/pol* expression cassette according to claim 22 wherein the 5' terminal end of said *gag/pol* gene lacks a retroviral packaging signal sequence.

24. A *gag/pol* expression cassette, comprising a promoter operably linked to a *gag/pol* gene, and a polyadenylation sequence, wherein a 3' terminal end of said *gag/pol* gene has been deleted without affecting the biological activity of integrase.

25. The *gag/pol* expression cassette according to claim 24 wherein a 5' terminal end of said *gag/pol* gene has been modified to contain codons which are degenerate for gag.

26. The *gag/pol* expression cassette according to claim 24 wherein said *gag/pol* gene lacks a retroviral packaging signal sequence.

27. The *gag/pol* expression cassette according to claims 24 to 26 wherein said 3' terminal end has been deleted upstream of nucleotide 5751 of Sequence ID No. 1.

28. The *gag/pol* expression cassette according to any one of claims 22 to 26 wherein said promoter is a heterologous promoter.

29. The *gag/pol* expression cassette according to claim 28 wherein said promoter is selected from the group consisting of CMV IE, the HSVTK promoter, RSV promoter, Adenovirus major-later promoter and the SV40 promoter.

30. The *gag/pol* expression cassette according to any one of claims 22 to 26 wherein said polyadenylation sequence is a heterologous polyadenylation sequence.

31. The *gag/pol* expression cassette according to claim 30 wherein said heterologous polyadenylation sequence is selected from the group consisting of the SV40 late poly A signal and the SV40 early poly A signal.

32. A *gag/pol* expression cassette, comprising a promoter operably linked to a *gag/pol* gene, and a polyadenylation sequence, wherein said expression cassette does not co-encapsidate with a replication competent virus.

33. An *env* expression cassette, comprising a promoter operably linked to an *env* gene, and a polyadenylation sequence, wherein no more than 6 consecutive retroviral nucleotides are included upstream of said *env* gene.

34. An *env* expression cassette, comprising a promoter operably linked to an *env* gene, and a polyadenylation sequence, wherein said *env* expression cassette does not contain a consecutive sequence of more than 8 nucleotides which are found in a *gag/pol* gene.

35. An *env* expression cassette, comprising a promoter operably linked to an *env* gene, and a polyadenylation sequence, wherein a 3' terminal end of said *env* gene has been deleted without effecting the biological activity of env.

36. The *env* expression cassette according to claim 35 wherein said 3' terminal end of said gene has been deleted such that a complete R peptide is not produced by said expression cassette.

37. The *env* expression cassette according to claim 36 wherein said *env* gene is derived from a type C retrovirus, and wherein the 3' terminal end has been deleted such that said *env* gene includes less than 18 nucleic acids which encode said R peptide.

38. The *env* expression cassette according to claim 36 wherein said 3' terminal end has been deleted downstream from nucleotide 7748 of Sequence ID. No. 1.

39. The *env* expression cassette according to any one of claims 33 to 38 wherein said promoter is a heterologous promoter.

40. The *env* expression cassette according to claim 39 wherein said promoter is selected from the group consisting of CMV IE, the HSVTK promoter, RSV promoter, Adenovirus major-later promoter and the SV40 promoter.

41. The *env* expression cassette according to any one of claims 33 to 38 wherein said polyadenylation sequence is a heterologous polyadenylation sequence.

42. The *env* expression cassette according to claim 41 wherein said heterologous polyadenylation is selected from the group consisting of the SV40 late poly A signal and the SV40 early poly A signal.

43. A packaging cell line, comprising a *gag/pol* expression cassette and an *env* expression cassette, wherein said *gag/pol* expression cassette lacks a consecutive sequence of greater than 8 consecutive nucleotides which are found in said *env* expression cassette.

44. A packaging cell line, comprising a *gag/pol* expression cassette according to claims 22 to 32, and an *env* expression cassette.

46. A packaging cell line, comprising a *gag/pol* expression cassette, and an *env* expression cassette according to claims 33 to 42.

46. A producer cell line, comprising a packaging cell line according to any one of claims 43 to 45, and a retroviral vector construct.

47. The producer cell line according to claim 46 wherein said retroviral vector construct is a retroviral vector construct according to any one of claims 1 to 21.

48. A producer cell line, comprising a *gag/pol* expression cassette, an *env* expression cassette, and a retroviral vector construct, wherein said *gag/pol* expression cassette, *env* expression cassette and retroviral vector construct lack a consecutive sequence of greater than 8 nucleotides in common.

49. A method of producing a packaging cell, comprising:

(a) introducing a *gag/pol* expression cassette according to claims 22 to 32 into an animal cell;

(b) selecting a cell containing a *gag/pol* expression cassette which expresses high levels of *gag/pol*;

(c) introducing an *env* expression cassette into said selected cell; and

(d) selecting a cell which expresses high levels of *env*, and thereby producing said packaging cell.

50. A method of producing a packaging cell, comprising:

(a) introducing an *env* expression cassette according to claims 33 to 42 into an animal cell;

(b) selecting a cell which expresses high levels of *env*;

(c) introducing a *gag/pol* expression cassette into said selected cell; and

(d) selecting a cell containing a *gag/pol* expression cassette which expresses high levels of *gag/pol*, and thereby producing said packaging cell.

51. A method of producing recombinant retroviral particles, comprising introducing a retroviral vector construct into packaging cell line according to claim 49 or 50.

52. The method according claim 51 wherein said retroviral vector construct is a retroviral vector construct according to any one of claims 1 to 21.

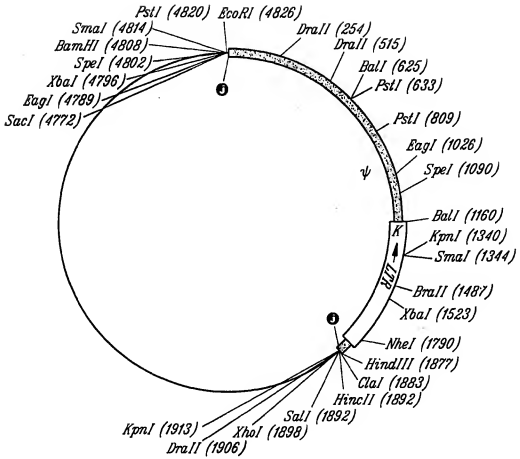

Fig. 1

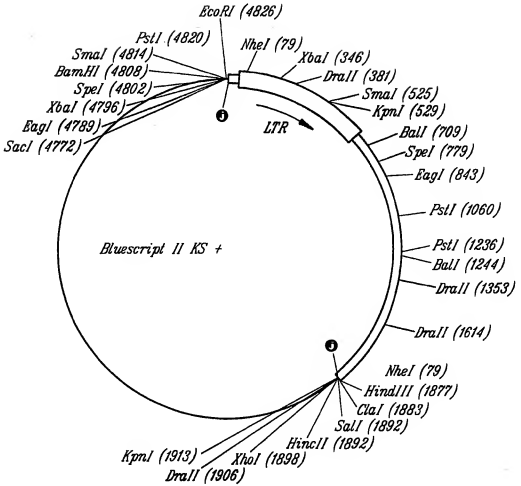

Fig. 2

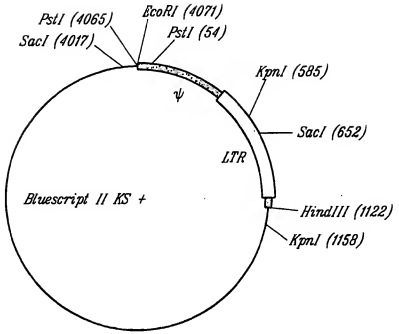

Fig. 3

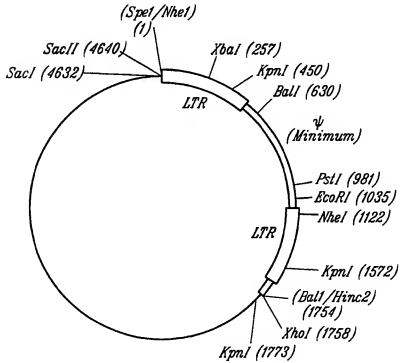

Fig. 4

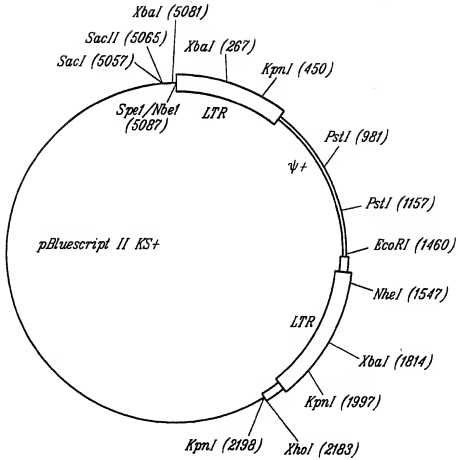

Fig. 5

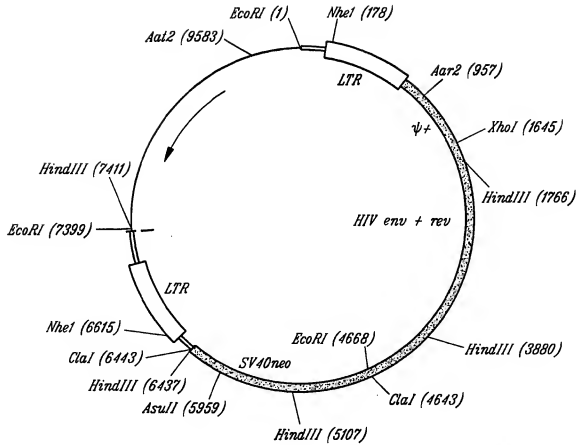

Fig. 6

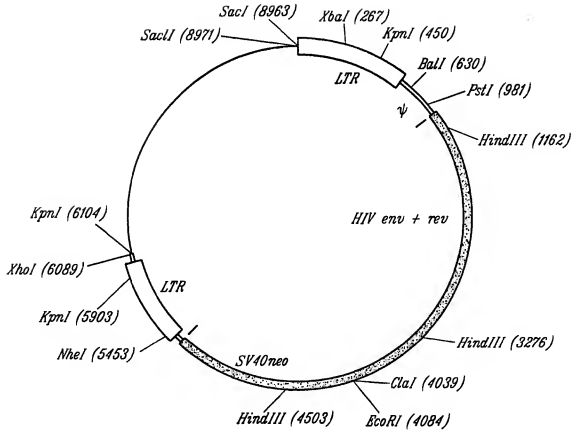

Fig. 7

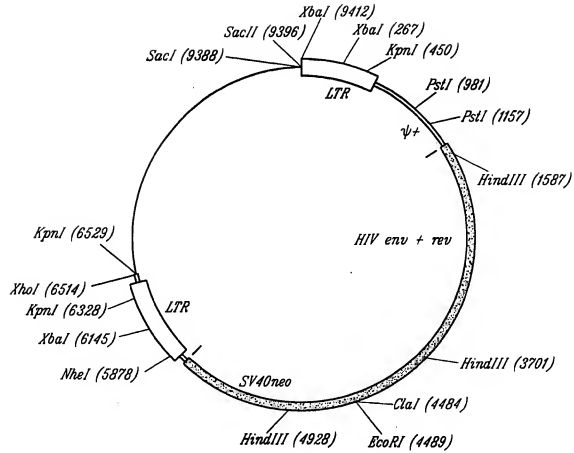

Fig. 8

```
1   ATG GGC CAG ACT GTT ACC ACT CCC TTA AGT TTG ACC TTA GGT CAC TGG AAA
1►  Met Gly Gln Thr Val Thr Thr Pro Leu Ser Leu Thr Leu Gly His Trp Lys

52  GAT GTC GAG CGG ATC GCT CAC AAC CAG TCG GTA GAT GTC AAG AAG AGA CGT
18► Asp Val Glu Arg Ile Ala His Asn Gln Ser Val Asp Val Lys Lys Arg Arg

103 TGG GTT ACC TTC TGC TCT GCA GAA TGG CCA ACC TTT AAC GTC GGA TGG CCG
35► Trp Val Thr Phe Cys Ser Ala Glu Trp Pro Thr Phe Asn Val Gly Trp Pro

154 CGA GAC GGC ACC TTT AAC CGA GAC CTC ATC ACC CAG GTT AAG ATC AAG GTC
52► Arg Asp Gly Thr Phe Asn Arg Asp Leu Ile Thr Gln Val Lys Ile Lys Val

205 TTT TCA CCT GGC CCG CAT GGA CAC CCA GAC CAG GTC CCC TAC ATC GTG ACC
69► Phe Ser Pro Gly Pro His Gly His Pro Asp Gln Val Pro Tyr Ile Val Thr

256 TGG GAA GCC TTG GCT TTT GAC CCC CCT CCC TGG GTC AAG CCC TTT GTA CAC
86► Trp Glu Ala Leu Ala Phe Asp Pro Pro Pro Trp Val Lys Pro Phe Val His

307 CCT AAG CCT CCG CCT CCT CTT CCT CCA TCC GCC CCG TCT CTC CCC CTT GAA
103► Pro Lys Pro Pro Pro Pro Leu Pro Pro Ser Ala Pro Ser Leu Pro Leu Glu

358 CCT CCT CGT TCG ACC CCG CCT CGA TCC TCC CTT TAT CCA GCC CTC ACT CCT
120► Pro Pro Arg Ser Thr Pro Pro Arg Ser Ser Leu Tyr Pro Ala Leu Thr Pro

                Nart (415)
409 TCT CTA GGC GCC
137► Ser Leu Gly Ala
```

Fig. 9

```
A TAT ATA TAT ATC GAT ACC  ATG GGG CAA ACC GTG ACT ACC CCT CTG TCC
                          ▶Met Gly Gln Thr Val Thr Thr Pro Leu Ser

 CTC ACA CTG GGC CAT TGG AAG GAC GTG GAA AGA ATT GCC CAT AAT CAA AGC
▶Leu Thr Leu Gly His Trp Lys Asp Val Glu Arg Ile Ala His Asn Gln Ser

 GTG GAC GTC AAA AAA CGC AGG TGG GTG ACA TTT TGT AGC GCC GAG TGG CCC
▶Val Asp Val Lys Lys Arg Arg Trp Val Thr Phe Cys Ser Ala Glu Trp Pro

 ACA TTC AAT GTT GGC TGG CCT AGG GAT GGA ACT TTC AAT CGC GAT CTG ATT
▶Thr Phe Asn Val Gly Trp Pro Arg Asp Gly Thr Phe Asn Arg Asp Leu Ile

 ACT CAA GTG AAA ATT AAA GTG TTC AGC CCC GGA CCC CAC GGC CAT CCC GAT
▶Thr Gln Val Lys Ile Lys Val Phe Ser Pro Gly Pro His Gly His Pro Asp

 CAA GTT CCT TAT ATT GTC ACA TGG GAG GCT CTC GCT TTC GAT CCA CCA CCT
▶Gln Val Pro Tyr Ile Val Thr Trp Glu Ala Leu Ala Phe Asp Pro Pro Pro

 TGG GTG AAA CCA TTC GTG CAT CCC AAA CCA CCT CCA CCC CTC CCA CCC AGC
▶Trp Val Lys Pro Phe Val His Pro Lys Pro Pro Pro Pro Leu Pro Pro Ser

 GCT CCT AGC CTG CCC TTG GAG CCC CCA CGA AGC ACA CCA CCC AGG AGC AGC
▶Ala Pro Ser Leu Pro Leu Glu Pro Pro Arg Ser Thr Pro Pro Arg Ser Ser

                                      NarI
 TTG TAC CCT GCT CTG ACC CCC AGC CTC GGC GCC AAA   CCT AAA C
▶Leu Tyr Pro Ala Leu Thr Pro Ser Leu Gly Ala Lys ? ???????
```

Fig. 10

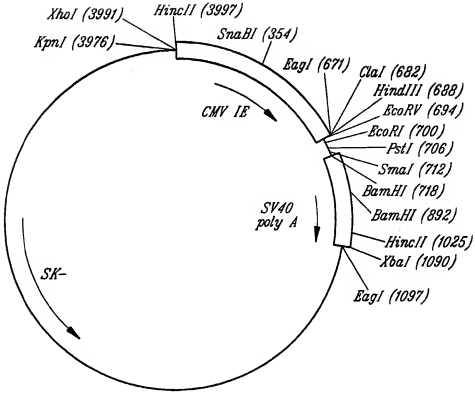

Fig. 11

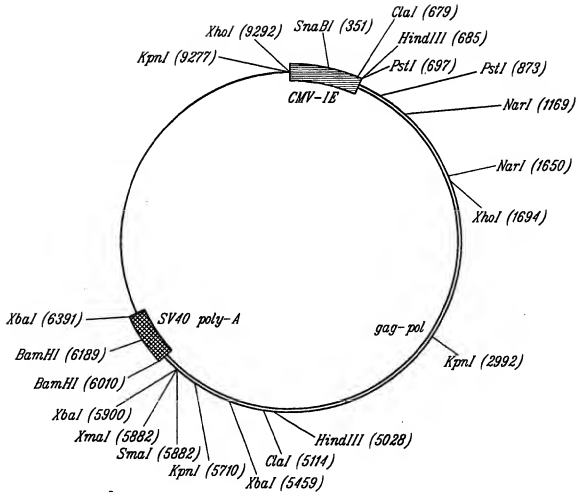

Fig. 12

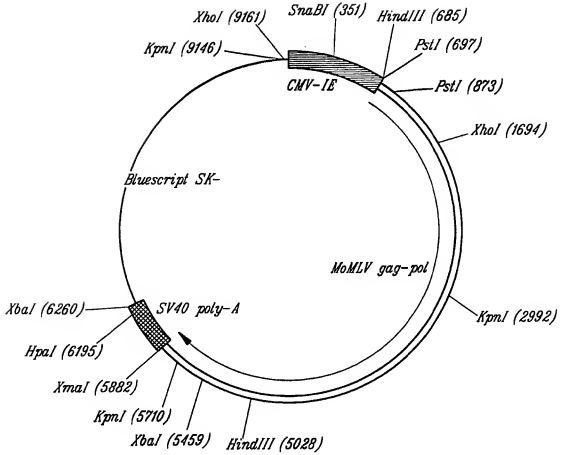

Fig. 13

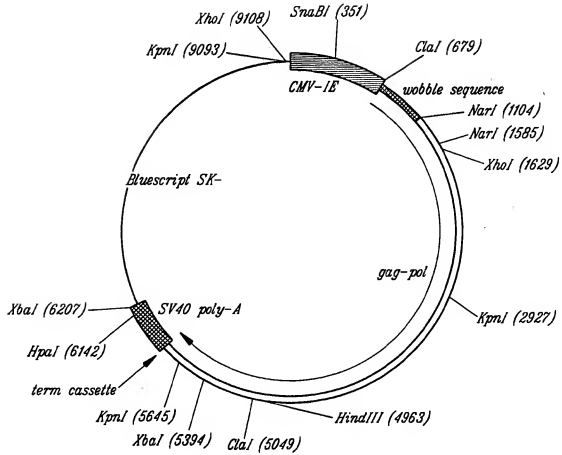

Fig. 14

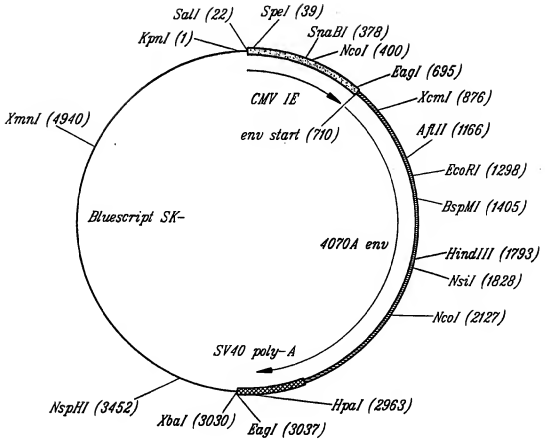

Fig. 15

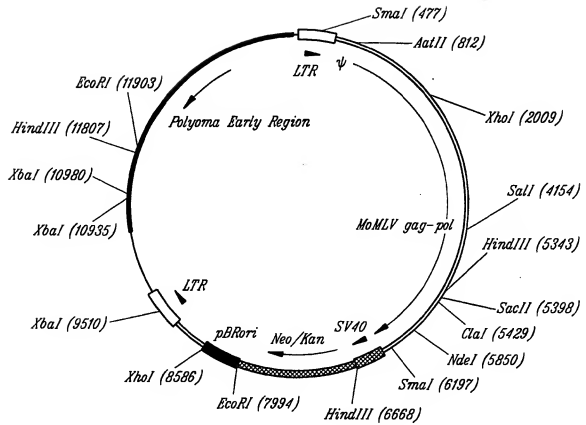

Fig. 16

Fig. 17A

VIRUS	SPECIES OF ISOLATION	TYPE[1]
AEV (Avian erthroblastosis virus)	chicken	C,X,T
ALV (avian leukosis virus)	chicken	C,N or X,N
AMV (avian myeloblastosis virus)	chicken	C,X,T
ASV (avian sarcoma virus)	chicken	C,X,T
BaEV (baboon endogenous virus)	baboon (*Papio ssp.*)	C,N,N
B1LN	*P. hamadryas*	
M7	*P. cynocephalus*	
M28	*P. cynocephalus*	
PP-1-Lu	*P. papio*	
TG-1-K	gelada	
BLV (bovine leukemia virus)	cow	C,X,N
BSV (bovine syncytial virus)	cow	S,X,N
CAEV (caprine arthritis-encephalitis virus)	goat	L,X,N
CERV-CI, CERV C-II	*Mus cervicolor*	C,N,N
CCC	cat	C,N,N
CPC-1	colobus monkey	C,N,N
CSRV (corn snake retrovirus)	corn snake	C,
CSV (chick syncytial virus)	chicken	C,X,N
DIAV (duck infectious anemia virus)	duck	C,X,N
DKV (deer kidney virus	black-tailed deer	C,N,N
DPC-1	agouti	C,N,N
EIAV (equine infectious anemia virus)	horse	C,X,N
ESV (Esh sarcoma virus)	chicken	C,X,T
FeLV (feline leukemia virus)	cat	C,N or X,N
FeSV (feline sarcoma virus)	cat	C,X,T
GA (Gardner-Arnstein)		
SM (McDonough)		
ST (Snyder-Theilen)		
FS-1	*Felis sylvestris* (wildcat)	C,N,N
FSFV (feline syncytium-forming virus	cat	S,X,N
FuSV (Fujinami sarcoma virus)	chicken	C,X,T
GALV (gibbon ape leukemia virus)	gibbon	C,X,N
GLV (goat leukoencephalitis virus)	see CAEV	
GPV (golden pheasant virus)	golden pheasant	C,N,N
HaLV (hamster leukemia virus)	hamster	C,N,N
IVL (induced leukemia virus)	chicken	C,N,N
LLV (lymphoid leukosis virus)	see ALV	
LPDV (lymphoproliferative disease of turkeys	turkey	C,X,T
M432	*Mus cervicolor*	B,N,N
M832	*Mus caroli*	B,N,N

[1] The first letter denotes classification: (B) B-type oncovirus; (C) C-type oncovirus; (D) D-type oncovirus; (L) lentvirus; (S) spumavirus. The second letter denotes origin: (N) enogenous; (X) exogenous; (R) recombinant. The third letter denotes ability to indice morphological transformation: (T) transforming (i.e., containing an *onc* sequence); (N) nontransforming; (?) unknown.

MAC-1	stumptail monkey	C,N,N
Maedi	sheep	L,X,N
MAV (myeloblastosis-associated virus)	chicken	C,X,N
MC29 (myelocytomatosis virus)	chicken	C,X,T
MCF (mink cell focus-inducing virus)	mouse	C,NR,N
MH2 (myelocytomatosis virus)	chicken	C,X,T
MiLV (mink leukemia virus)	mink	C,N,N
MLV (murine leukemia virus)	mouse	C,X or N,N
Ab (Abelson)		C,X,T
Fr (Friend)		C,X,N
Graffi		C,X,N
Gross		C,N,N
Ki (Kirsten)		C,X,N
Mo (Moloney)		C,X,N
Ra (Rauscher)		C,X,N
MMC-1	rhesus monkey	C,N,N
MMTV (mouse mammary tumor virus)	mouse	B,X or N,N
MPMV (Mason-Pfizer monkey virus)	rhesus monkey	D,X,N
MSV (murine sarcoma virus)	mouse	C,X,T
BALB		
FBJ (Finkel-Biskis-Jinkins)		
FBR		
Gz (Gazdar)		
Ha (Harvey)		
Ki (Kirsten)		
Mo (Moloney)		
MPV[1] (myeloproliferative)		
OS2 (osteosarcoma)		
MyLV (myeloid leukemia)	mouse	C,X,N
OK10 (myelocytomatosis virus)	chicken	C,X,T
OMC-1	owl monkey	C,N,N
PK-15	pig	C,N,N
PO-1-Lu	langur	D,N,N
PPV (progressive pneumonia virus)	sheep	L,X,N
PRCII, PRCIV (Poultry Research Centre)	chicken	C,X,T
R-35	rat	C,X?,T
RaLV (rat leukemia virus)	rat	C,X,N
RaSV (rat sarcoma virus)	rat	C,X,T
RAV-*n* (Rous-associated virus)	see ALV	
RAV-0 (Rous-associated virus 0)	chicken	C,N,N
RAV-60 (Rous-associated virus 60)	chicken	C,R,N
RAV-61 (Rous-associated virus 61)	ring-necked pheasant	C,R,N
RD114	cat	C,N,N
REAV (reticuloendotheliosis-associated virus)	turkey	C,X,N

Fig. 17B

REV (reticuloendotheliosis virus)	birds	C,X,N
REV-T (reticuloendotheliosis virus-transforming	turkey	C,X,T
RIF (Rous interference factor)	see ALV	
RPL-*n* (Regional Poultry Laboratory)	see ALV	
RPV (ring-necked pheasant virus)	ring-necked pheasant	C,R,N
RSV (Rous sarcoma virus)	chicken	C,X,T
B77 (Bratislava)		
BH (Bryan high titer)		
BS (Bryan standard)		
CZ (Carr-Zilber)		
EH (Engelbreth-Holm)		
HA (Harris)		
PR (Prague)		
SR (Schmidt-Ruppin)		
SFV-*n* (simian foamy virus)	monkey	S,X,N
SFFV (spleen focus-forming virus)	mouse	C,X, or R,N or T
Friend		
MPV		
Rauscher		
SiSV (simian sarcoma virus)	see SSV	
SLV (simian lymphoma virus)	see GALV	
SMRV (squirrel monkey retrovirus)	squirrel monkey	D,N,N
SMV (simian myelogenous leukemia virus)	see GALV	
SSAV (simian sarcoma-associated virus)	woolly monkey	C,X,N
SSV (simian sarcoma virus)	woolly monkey	C,X,T
TRV-1	tree shrew	C,N,N
UR-*n* (University of Rochester)	chicken	C,X,T
Vand C-1	tree mouse	C,N,N
Visna	sheep	L,X,N
VRV (viper retrovirus)	Russell's viper	C,N,?
WMV (woolly monkey virus)	see SSV	
WoLV (woolly monkey leukemia virus)	see SSAV	
Y73 (Yamaguchi 73)	chicken	C,X,T

Fig. 17C

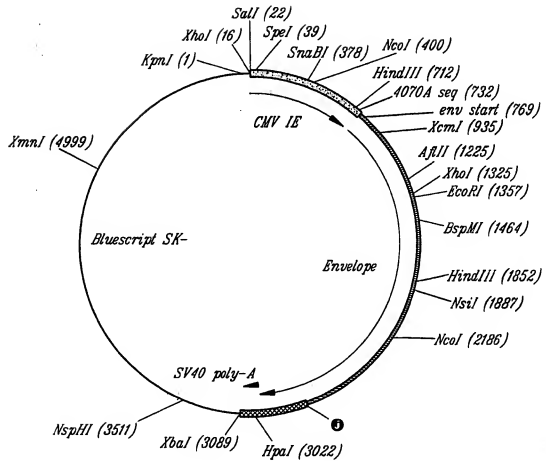

Fig. 18

CMV Promoter – wobble gag – SVneo – LTR

Fig. 19A

CMV Promoter – normal gag – SVneo – LTR

Fig. 19B